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Developmental Studies and Laboratory Investigations Conducted by Veterinary Services Laboratories Fiscal Year 1974

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DEVELOPMENTAL STUDIES AND LABORATORY INVESTIGATIONS CONDUCTED BY VETERINARY SERVICES DIAGNOSTIC LABORATORIES FISCAL YEAR 1974

ANAPLASMOSIS

A MICROTITER TECHNIQUE FOR THE COMPLEMENT FIXATION TEST FOR ANAPLASMOSIS. Martin, W. H. and Ritchie, W. H. Proc. 77th Ann. Mtg. of the USAHA, Oct. 1973, pp. 582-592. (Abstract of Published Report)

A microtiter technique has been developed for the diagnosis of bovine anaplasmosis utilizing the highly purified antigen initially designed for use in the card test system. The antigen consists of a suspension of *Anaplasma marginale* bodies without the extraneous materials present in previous antigens.

Comparison of the standard tube technique with the new microtiter method on 7,120 samples showed agreement between them to be 96 percent. The results were identical on all positive (4+) samples; the discrepancies appeared only on those samples showing suspicious (1+, 2+, 3+) reactions on the tube, but were negative on the microtiter test. All samples negative on tube tests were negative on microtiter. This small discrepancy is attributed to the use of the new highly purified antigen.

BLUETONGUE

BLUETONGUE VIRUS IN CATTLE: COMPLEMENT-FIXING ANTIBODY RESPONSE AND VIREMIA IN EXPERIMENTALLY INFECTED ANIMALS. Pearson, J. E., Carbrey, E. A., and Gustafson, G. A. Proc. 77th Ann. Meeting USAHA, 1973, pp. 524-531. (Abstract of Published Report)

The persistence of bluetongue virus and complement-fixing antibody in calves was evaluated. Eighteen calves were inoculated with three strains of virus: six with the BT-8 strain, six with the 62-45S strain, and six with the 63-83B strain. One calf that was inoculated with the 63-83B strain did not develop antibody and virus was not reisolated so it was assumed this calf was not infected. Complement-fixing antibody was detected in the other 17 calves between post-inoculation day (PID) 19 and 28. The calves inoculated with 62-45S and 63-83B were negative for complement-fixing antibody by PID 110 while the calves receiving BT-8 were still positive at PID 162 when the experiment was terminated.

Virus was isolated by sheep inoculation from 15 of the 17 calves at PID 30. One calf died of ruminal tympany at PID 32 leaving 16 calves from the remainder of the experiment. At PID 60 only one calf was positive for virus isolation and it was negative on the next isolation attempt at PID 113. Complement-fixing antibody was detected in all calves at the time virus isolations were made. Statistical evaluation, based on 16 infected calves, indicated at least 82.9 percent of the bluetongue infected cattle will not have a viremia that persists past PID 113.

BOVINE VIRUS DIARRHEA

Chemically Induced Stress in Experimental Bovine Virus Diarrhea-Mucosal Disease Infection. Tamoglia, T. W. and Hanson, S. K. (Project Report)

Summary

Two bovine virus diarrhea (BVD) seronegative calves were given 20 mg of Dexamethasone (DXM) daily for 10 days. They were exposed to BVD challenge virus by intranasal administration and both developed classical BVD-mucosal disease signs. Another calf vaccinated with BVD vaccine 28 days before chemical stress and challenge of immunity did not develop clinical signs.

Introduction

The BVD calf challenge virus supplied to licensees by Veterinary Services Laboratories for Master Seed Virus antigenicity testing *(1)*[1] causes a diphasic temperature elevation response and leucopenia in BVD seronegative calves *(2)*. It does not, however, produce the typical feedlot BVD-mucosal disease syndrome characterized by scouring, depression, excessive salivation, anorexia, dehydration, conjunctivitis, and ulcerations in the mucous membrane of the oral cavity *(3)* when administered to healthy calves. This report concerns studies of response to the BVD challenge virus in three chemically stressed calves. Two were BVD seronegative and one was vaccinated with a BVD vaccine before being stressed and challenged.

Materials and Methods

Experimental Calves

Three BVD seronegative calves, approximately six months of age, were used in the experiment.

Challenge Virus

APHIS-VS-BL BVD challenge virus 70-2 is a third cell culture passage of dried spleen identified as NY-1 C 597R 14-IX-70. The procedure for intranasal administration of the challenge virus has been previously described *(4)*.

Chemical Stress Agent

Azium Solution—a brand of Dexamethasone (DXM) containing 2 mg per cc.

Serology

Serum antibody titers were determined by the beta procedure with 100-500 $TCID_{50}/0.1$ ml. BVD reference virus used as the test dose.

Blood Studies

Total white blood cell (WBC) counts were determined by the unopette-hemacytometer procedure.

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[1] Numbers in parentheses refer to References at the end of this report.

Clinical Observations

Each calf was examined daily, rectal temperatures taken, and WBC counts made.

Experiment Number 1

The first experiment was done with one seronegative calf. The calf was given 10 ml (20 mg) of Dexamethasone daily for 10 days. Challenge virus was administered intranasally on day 6. Necropsy was performed on day 11 postchallenge (PC).

Experiment Number 2

This experiment involved two BVD seronegative calves. One calf was vaccinated intramuscularly (IM) with 2.0 ml of BVD vaccine. The vaccine, prepared with the NADL strain of virus, had a log 10 titer of 4.6 per 2 ml. The other calf received no vaccine but remained in contact with the vaccinated calf.

Starting on day 28 postvaccination (PV), both calves were given 10 ml (20 mg) of DXM IM daily for 10 consecutive days.

On day 32 PV, blood was taken for serum antibody assay and the immunity of both calves was challenged. Temperatures were taken and WBC counts determined daily through day 14 PC. Necropsy of the control calf was performed on day 14 PC and day 15 PC on the vaccinated calf.

Blood for serum antibody assay was taken from the vaccinated calf on days 9 and 15 PC.

Results

Experiment Number 1

The WBC count of this calf on day 0, the day DXM treatment was started, was 13,600. On day 2, the WBC count was 17,190; on day 5, 10,900; and on day 16, 3,500. Immunity was challenged on the fifth day of the DXM treatment. The temperature was 101.6° F. on day 0; on day 5, 101.6° F.; on day 12, 106° F.; and on day 16, 103.8° F. (fig. 1).

There was a diarrhea starting on day 5 PC and continuing through day 16. Blood appeared in the feces on day 5 PC and progressed to a haemorrhagia on day 11 PC. Starting on day 7 PC, the clinical signs observed included anorexia, weakness, dehydration, bloody scours, and serosanguinous nasal discharge. The calf was down and too weak to stand when euthanized on day 11 PC.

The lesions observed at necropsy included ulcerations of the lingual mucosa, ecchymoses on the surface of the spleen, urinary bladder, and gall bladder, hemorrhage in the small colon, and congestion of mediastinal lymph nodes. Other organs appeared normal macroscopically.

Experiment Number 2

Control Calf—The WBC count of control calf No. 9 on day 28 PV, the day DXM treatment started, was 8,700 on day 32, the WBC count was 7,200 and day 46 (14 days PC) 1,100.

Immunity was challenged on day 32 PV. The temperature rose from 101.8° F. on day of challenge to 107.4° F. on day 12 (fig. 2).

The clinical behavior of this nonvaccinated control calf was similar to that of nonvaccinated calf in the previous experiment.

The lesions observed at necropsy included ulcerations of the lingual mucosa; ecchymoses in the myocardium, reticulum, and small colon; edema of the spiral folds of fundus gland region of the abomasum; ulceration on the ileocecal valve and blood filled small colon and cecum.

Vaccinated Calf—The WBC count of this calf on day 28 PV, the day DXM treatment started, was 7,700. On day 32, the day of challenge, the WBC count was 10,100; and on day 46 (15 days PC), WBC count was
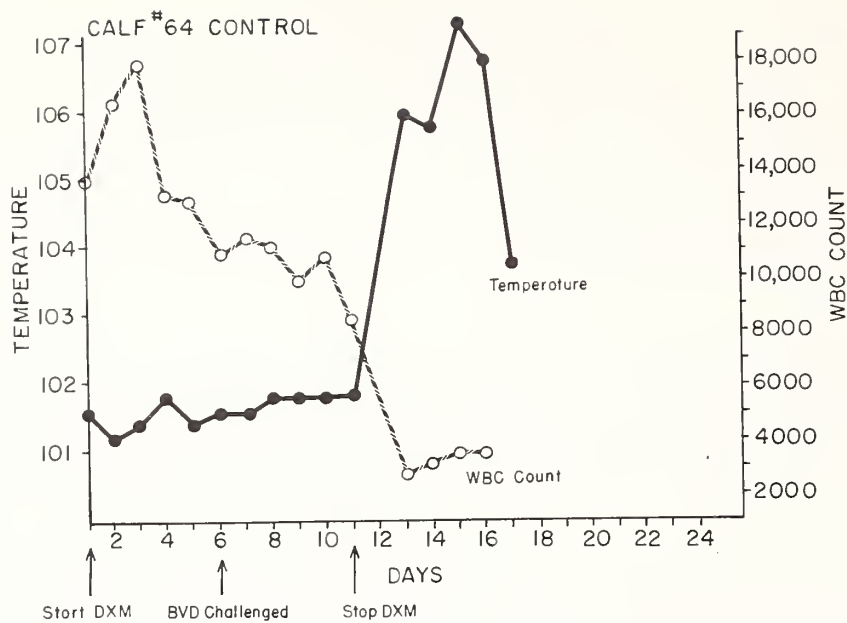
Figure 1.—Temperature and White Blood Cell Response to BVD Challenge in Chemically Induced Stress - Seronegative Calf.
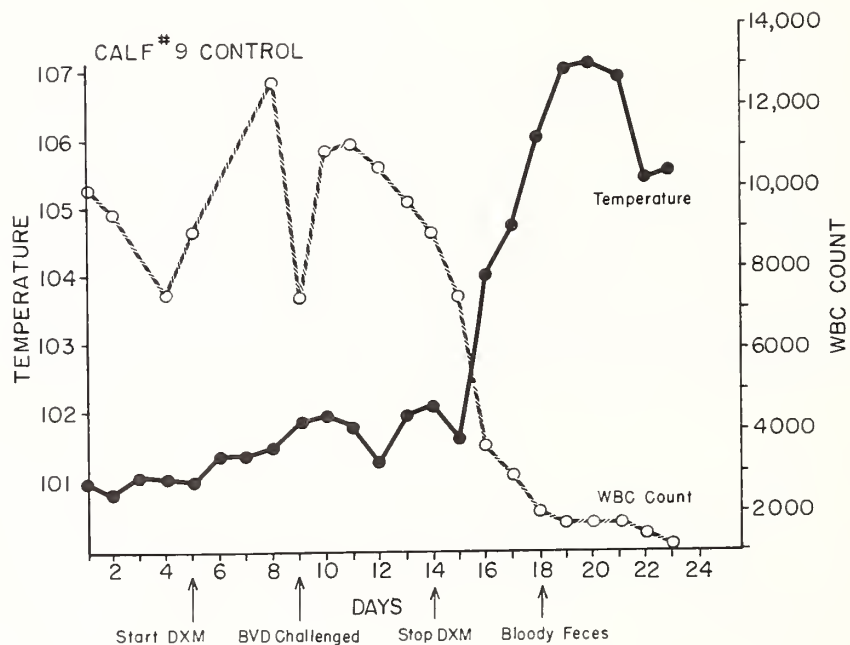
Figure 2.—Temperature and White Blood Cell Response to BVD Challenge in Chemically Induced Stress.

8,200 (fig. 3). The temperature of this calf remained within the normal range throughout the 15-day PC period.

The clinical behavior of this calf remained normal. While the calf appeared to be somewhat depressed on days 10-14 PC, the appetite was not impaired. The consistency of the stool remained normal, but occasionally a slightly blood tinged flob of mucus (marble size) appeared in the feces. BVD virus could not be isolated from this material.

At necropsy all tissues and organs appeared normal. On the day (PV 32) the immunity was challenged the BVD serum titer was 1:126. This rose to 1:320 on PC day 9 and >1:2000 by PC day 15.
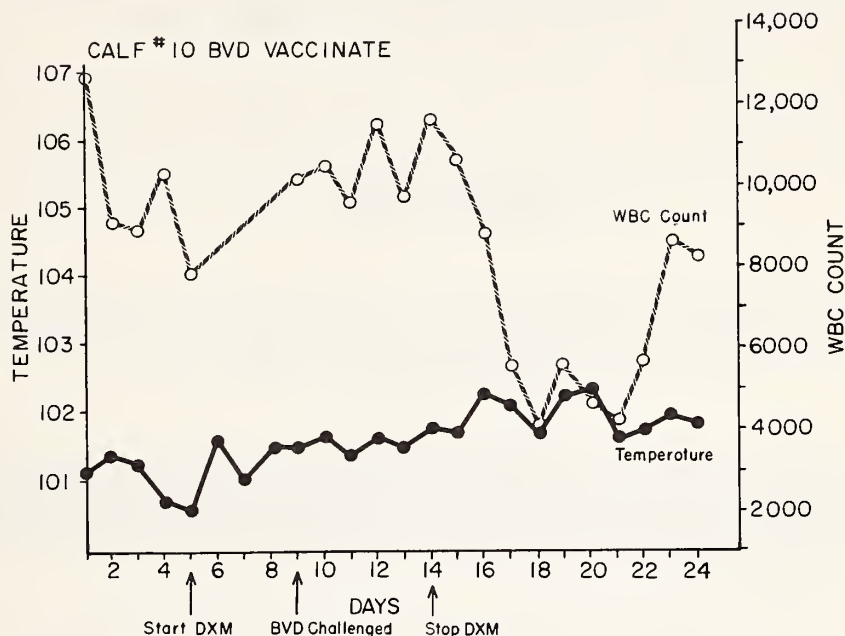
Figure 3.—Temperature and White Blood Cell Response to BVD Challenge in Chemically Induced Stress - BVD Vaccinated Calf.

Discussion

The only evidence of BVD infection in the vaccinated calf following challenge was descending WBC count and the ascending serum antibody titer. The BVD mucosal disease syndrome observed in field cases of the disease did not occur. Neither did severe systemic reaction occur following challenge as the temperature remained within the normal range and no overt clinical signs developed. The two nonvaccinated calves developed severe BVD mucosal disease following challenge. It is common knowledge that corticosteroids are antiinflammatory and, immunosuppressant to bacterial infections. However, the DXM used in this experiment had little, if any, effect on the established BVD immunity and no apparent effect on the antigenic response following challenge.

Conclusions

1. Severe BVD mucosal disease can be produced in Dexamethasone stressed BVD seronegative calves by intranasal administration of Veterinary Services Laboratories BVD challenge virus.
2. A calif vaccinated with BVD vaccine and allowed 28 days to develop immunity was protected against challenge following chemically induced stress.

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This information is useful for the assay of BVD vaccine efficacy because we now have a procedure for measuring the degree of protection afforded against the severe form of the disease as it occurs under feedlot conditions.

References

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2. Anon. Report on Veterinary Biologics Division BVD Challenge Virus, USDA, APHIS, VS. December 3, 1970.
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4. Anon. Instruction for Administering BVD Challenge Virus, Veterinary Biologics Division, Large Animal Products/Virology, USDA, APHIS, VS. December 4, 1970.

BRUCELLOSIS

SURVEY OF SELECTED CARNIVORE AND OPOSSUM SERUMS FOR AGGLUTININS TO BRUCELLA CANIS. Hoff, G. L., Bigler, W. J., Trainer, D. O., Debbie, J. G., Brown, G. M., Winkler, W. G., Richards, S. H. and Reardon, M., Jr. AVMA, Vol. 165, No. 9, Nov. 1974, pp. 830-831. (Abstract of Published Report)

A serologic survey for *Brucella canis* was conducted in opossums and seven species of wild carnivores collected from five States. A total of 770 serums were tested. One raccoon, one bobcat, one red fox, and two coyotes were seropositive. Results for 16 coyote serums were considered inconclusive. Reactors were not detected among striped skunks, gray foxes, wolves, or opossums.

THE UTILIZATION OF BRUCELLA ABORTUS CULTURING AND BIOTYPING RESULTS IN THE EPIZOOTI-OLOGIC INVESTIGATION OF BOVINE BRUCELLOSIS. Luchsinger, D. W., Angus, R. D., Gue. C. S. and Anderson, R. K. Proc. 77th Ann. Meeting of the USAHA, 1973, pp. 85-99. (Abstract of Published Report)

During an 8-year period, 730 *Brucella abortus* isolates were obtained from 331 cattle herds in Minnesota. Epizootiologic determinations of sources of infection and modes of transmission were significantly increased when culturing and biotyping information was included as part of the total investigation. Results of isolation attempts were used to monitor seriologic diagnoses and increase confidence in correctly evaluating herd status. Program modifications based on epizootiologic findings were recommended following evaluations of data from both infected and non-infected herds. There is an urgent need to include bacteriologic culture procedures as an integral part of the epizootiological investigations used to confirm that brucellosis eradication has been achieved in a State or country.

Three *B. abortus* biotypes were isolated from Minnesota cattle. Biotype 1 was predominant and widely distributed in the State. Biotype 4 was confined to the central area of the state but expanded its distribution by movement of infected and exposed cattle. Biotype 2 was usually associated with interstate cattle importation. Isolates indistinguishable from Strain 19 were geographically scattered in the State.

The distribution of *B. abortus* biotypes isolated from cattle in the United States is reviewed and compared with the data from Minnesota.

ENCEPHALITIS

A COMPARISON OF CLINICAL MANIFESTATIONS AND PATHOLOGY OF THE EQUINE ENCEPHALITIDES: VEE, WEE, EEE. Miller, L. D., Pearson, J. E. and Muhm, R. L. Proc. of the 77th Ann. Mtg. of the USAHA, 1973, pp. 629-631. (Abstract of Published Report)

The clinical signs and lesions seen in ponies inoculated with Venezuelan equine encephalomyelitis virus (VEE) were compared to those caused by Eastern (EEE) and Western (WEE) infection. The clinical signs were similar in all three diseases and included pyrexia, depression, inappetance and somnolence. Head pressing, convulsions and paddling occurred in some cases. Circulating leukocyte levels were decreased initially in those animals examined hematologically.

Gross lesions were limited to congestion of the brain and meninges. Ecchymotic hemorrhages of traumatic origin were seen in two cases with terminal convulsions. Microscopic lesions produced by EEE were much more extensive and severe than those of WEE. In EEE, neutrophils were the predominating inflammatory cell type and large areas of the brain, especially the cerebral cortex were affected. Primarily, lymphocytes and other mononuclear cells were seen in WEE. The lesions of VEE were intermediate in both extent and severity.

ERYSIPELAS

COMPARISON OF A FLUORESCENT ANTIBODY TECHNIQUE AND CULTURAL METHOD FOR THE DETECTION OF ERYSIPELOTHRIX RHUSIOPATHIAE IN PRIMARY BROTH CULTURES. Harrington, R., Jr., Wood, R. D., Hulse, D. C. Am. J. Vet. Res., Vol. 35, No. 3, Mar. 1974, pp. 461-462. (Abstract of Published Report)

A fluorescent antibody (FA) technique was compared with a cultural method for the detection of *Erysipelothrix rhusiopathiae* in 1,412 broth cultures. There was agreement between the FA technique and cultural method on 1,089 cultures. The two methods did not agree on 323 cultures.

ERYSIPELAS IMMUNIZING PRODUCT REVIEW. Bairey, M. H. and Vogel, J. H. Proc. 77th Ann. Meeting USAHA, 1973, pp. 340-344. (Abstract of Published Report)

Over the 4-1/2 years, six serials of vaccine have been evaluated for potency in swine. At least one serial of each type of vaccine and at least one serial from each producer. Five serials protected at least three of four pigs and one serial protected only two of four for 83.3 percent satisfactory testing rate.

During this same period of time 49 serials of bacterin were assayed in swine. Forty-six of these serials protected at least three of four pigs while three did not. This was a 93.9 percent satisfactory testing rate. Twenty-six serials of bacterin were assayed by the mouse test with 20 satisfactory and 6 unsatisfactory for only a 76.9 percent satisfactory testing rate.

One hundred and thirteen serials of antiserum have been assayed in mice with 93 serials being satisfactory for a satisfactory testing rate of 82.3 percent.

HOG CHOLERA

BORDER DISEASE-LIKE SYNDROME IN LAMBS: ANTIBODIES TO HOG CHOLERA AND BOVINE VIRAL DIARRHEA VIRUSES. Osburn, B. I., Clarke, G. L., Stewart, W. C. and Sawyer, M. S., Jr. AVMA, Vol. 163, No. 10, Nov. 1973, pp. 1165-1167. (Abstract of Published Report)

Serum samples were collected from fetal and newborn lambs with a naturally occurring border disease-like syndrome and from lambs inoculated with brain-spleen suspensions obtained from affected lambs. Serum neutralization (SN) tests for antibodies to bovine viral diarrhea (BVD) and hog cholera viruses and the single radial immunodiffusion assay for quantitating immunoglobulin G (IgG) were performed. In precolostral samples, serum IgG content was less than 0.10 mg./ml. in control lambs and ranged from 0.10 to 1.1 mg./ml. in affected lambs; postcolostral values exceeded 7.5 mg./ml. Serum-neutralizing antibodies to hog cholera were detected in all of 13 samples examined, the titers ranging from 1:4 to 1:1,024. Serum-neutralizing antibodies to BVD were in 8 of 13 samples that had titers to hog cholera.

MICROTITER TECHNIQUES

MICROTITER AND AUTOMATED SEROLOGIC TECHNIQUES FOR DIAGNOSTIC VIROLOGY. Carbrey, E. A., Downing, D. R., Snyder, M. L., Wessman, S. J., and Gustafson, G. A. Proc. 77th Ann. Mtg. of the USAHA, Oct. 1973, pp. 553-562. (Abstract of Published Report)

Microtiter serologic techniques provide a reasonably accurate and extremely economical means of confirming infections with animal diseases. Although precise and tedious work is involved in their application, the procedure is well accepted by technicians.

Automated procedures such as provided by the Auto Analyzer system can eventually place low-cost serology within reach of every diagnostic laboratory.

MICROTITER SEROLOGIC TECHNIQUES FOR DIAGNOSTIC BACTERIOLOGY. Ellis, E. M. Proc. 77th Ann. Mtg. of the USAHA, Oct. 1973, pp. 653-581. (Abstract of Published Report)

Microtiter serologic techniques have proved to be a saving in time and materials. Their introduction into the diagnostic laboratory has made possible the testing of considerably more specimens than would be possible using previous test tube techniques.

CHARACTERIZATION OF THE FATTY ACIDS OF MYCOBACTERIUM BOVIS BY GAS-LIQUID CHROMA-TOGRAPHY. Thoen, C. O., Jarnagin, J. L. and Champion, M. (Project Report)

Introduction

Previous studies have revealed that gas-liquid chromatographic analyses of the methyl esters of cellular fatty acids were of value in detecting similarities and differences of certain mycobacteria. *(3-5)*[1] The objectives of this study were to obtain information on the celluar fatty acid composition of *Mycobacterium bovis* isolated from cattle in different geographical regions of the United States and to evaluate the feasibility of using gas-liquid chromatographic analyses for identifying *M. bovis* in the veterinary diagnostic laboratory.

In this report information is presented on the fatty acid composition of 4 strains of *M. bovis* isolated from cattle in Florida, Illinois and Texas, Two strains of *M. avium* and 1 strain of *M. tuberculosis* were included for comparison. The organisms were identified by morphologic, biochemical and drug sensitivity tests *(1)*.

Materials and Methods

Each of the strains of mycobacteria were subcultured on Lowenstein-Jensen medium for 14 days. A loopful of growth was transferred to 300 ml of Proskauer and Beck medium with albumin and pyruvage in 1,000 ml Erlenmeyer flasks and incubated at 37° C for 28 days. Duplicate cultures of each strain were made and duplicate analyses were conducted on each specimen. Flasks of uninoculated medium were included as controls. Smears and sub-cultures were made at the time of harvesting to detect contamination.

The mycobacterial cells were harvested by centrifugation for 20 minutes at 3,000 revolutions per minute. The cellular fatty acids were extracted as described previously *(4)* The fatty acids were esterified with diazomathane by the method of Schlenk and Gellerman *(2)*.

The methyl esters of the bacterial fatty acids were analyzed by gas chromatography; a hydrogen flame ionization dector (F-M model 500-1609) was used. The specimens were injected directly onto the chromatographic column. The columns were 1.83 m long and were packed with diethylene glycol succinate (15 percent) on siliconized chromosorb G-AW (60-80 mesh). The column was operated isothermally at a temperature of 200° C. with a detector temperature of 275° C. Fatty acids in the bacterial specimens were identified by comparison with retention times of known standards containing saturated fatty acids (fig. 1).

Results and Discussion

The fatty acid profiles of *M bovis, M. tuberculosis,* and *M. avium* as determined by gas-liquid chromatographic analyses contained saturated fatty acids ranging from C_8 to C_{24} (table 1). The cellular fatty acid composition of each of the *M. boxis* strains examined were similar. The C_{16} fatty acids were present in the largest amount (more than 30 percent) of lipid in all the isolates examined. C_{19} branched chain fatty acid tentatively identified as tuberculosteric acid accounted for more than 18 percent of the fatty acid composition. The fatty acid profile of *M. bovis, M. tuberculosis* and *M. avium* serotype two strains were alike, but different from the profiles of *M. kansasii* previously reported *(4)*. The information on fatty acid composition of *M. bovis* is limited to four strains isolated from cattle in different geographical areas; however, the results indicate that each of the strains contain similar quantities of fatty acids C_8 - C_{20}: No characteristic fatty acid components were detected in *M. bovis* which would be useful in differentiating them from *M. tuberculosis* or other nonphotochromogenic slowly growing mycobacteria.

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[1] Numbers in parentheses refer to References at the end of this report.
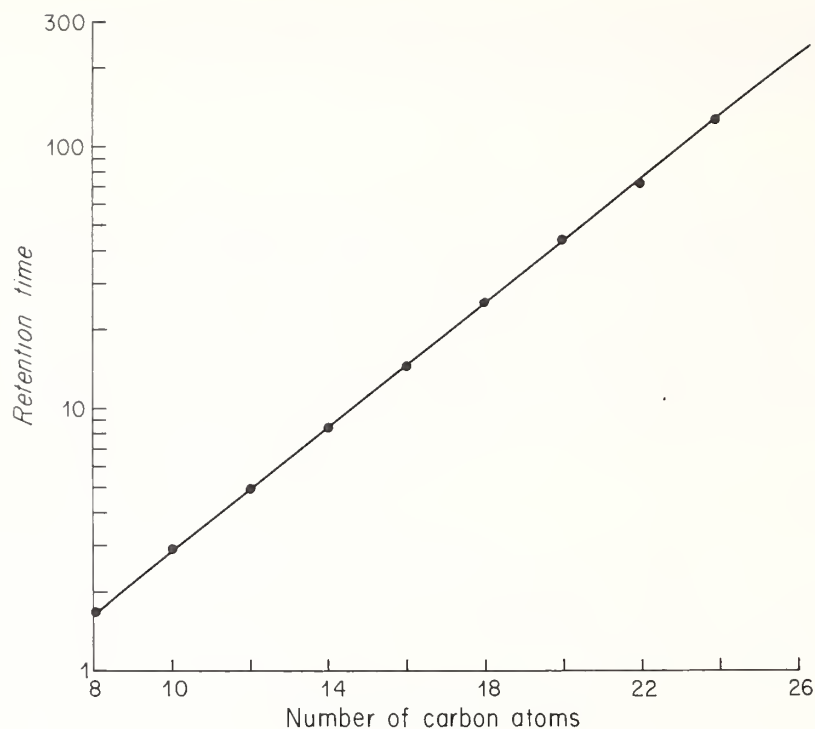
Figure 1.—Graph of log of retention time of fatty acids plotted against carbon number of saturated fatty acids on a polyester column.

Table 1.—*Percentage of cellular fatty acid composition of M. Bovis,*
M. Tuberculosis and of M. Avium

Carbon no.	Retention time (min) of methyl esters of fatty acids on DEGS[1]	Fatty acid composition(%)		
		M. bovis	M. tuberculosis	M. avium
8:0	1.7	T	T	T
10:0	2.9	T	T	T
11:0	3.7	T	T	T
12:0	5.0	T	T	T
14:0	8.4	3.5	3.8	5.3
15:0	10.7	T	T	T
16:0	14.5	32.2	35.4	36.2
16:1	17.3	4.9	5.4	6.5
17:0	19.2	2.6	2.8	2.1
18:0	24.8	8.8	11.4	9.6
18:1	28.8	5.7	6.7	6.0
19:0	32.4	7.9	6.1	7.2
19B[2]	29.4	22.2	20.1	18.6
20:0	42.4	2.8	3.2	2.5
22:0	72.0	3.1	1.7	2.0
24:0	126.0	3.3	2.4	2.8

[1] DEGS - Diethylene glycol succinate coated on Chromosorb G-AW.
[2] Tentatively identified as tuberculosteric acid.
T - Trace amount.

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5. Thoen, C. O., Karlson, A. G. and Ellefson, R. D. 1971 Comparison by Gas-Liquid Chromatography of the Fatty Acids of *Mycobacterium avium* and Some Other Nonphotochromogenic Mycobacteria. Appl. Microbiol. *22*:560-563.

CHICKEN LIVER MEDIUM, Richards, W. D. (Project Report)

Summary

An infusion of tuberculous chicken livers was used in this experiment to prepare Tuberculous Chicken Liver (TCL) medium which was compared to other mycobacteriology media. In 37 trials using bovine or porcine tissues, isolation was accomplished on regular medium only in three instances and on TCL medium only in one instance.

Introduction

Pathogenic mycobacteria generally grow slowly and occasionally fail to grow on laboratory media. To improve isolation efficiency, a great variety of media supplements have been tried since Koch prepared the first coagulated serum medium. Probably the most notably successful supplement is mycobactin, an alcoholic extract of *M. phlei*. In this experiment, an infusion of tuberculous chicken livers was incorporated into a whole egg media. The isolation efficiency of the TCL medium was compared to that of four other types of culture media.

Materials and Methods

Healthy leghorn chickens were inoculated intraperitoneally with 0.1 mg of viable *M. avium* serotype 2 cells. The livers of the infected chickens were collected within 16 hours after their death and sterilized by autoclaving. They were weighed, sliced, and ground in a minimum amount of sterile distilled water. The livers were boiled for 1 hour in a covered flask while maintaining a minimum amount of fluid covering the tissues.

The infusion was filtered through sterile unbleached muslin followed by sufficient sterile distilled water to provide a final volume equivalent to 1 ml of infusion for each gram of liver. The infusion was refrigerated overnight and the layer of fat removed. The chemicals and dyes shown in table 1 were dissolved in separate portions of infusion, autoclaved for 15 min. at 121 C and mixed in a sterile flask. Whole eggs were added aseptically, mixed

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thoroughly and dispensed into 20 x 125 mm test tubes. Coagulation and final sterilization was accomplished by inspissation at 85 C for 50 minutes.

Table 1.—Composition of Tuberculous Chicken Liver Medium

Magnesium Citrate	0.2	gm
Sodium Pyruvate	1.0	gm
Potassium Dihydrogen Phosphate $KH_2(PO4)_2$	0.4	gm
Disodium Phosphate Na_2HPO	Q.S. to ph 6.4	
Glycerine	1.4	ml
TCL Infusion	78.0	ml
Whole Egg..............................	2	
Crystal Violet	.01	gm
Malachite green (1% aqueous solution).........	0.8	ml

Two slants of TCL medium were inoculated in addition to the eight slants of regular media, using sediment inoculum from 37 mycobacteriology tissue specimens, (26 bovine, 11 porcine). All slants were incubated at 37°C for 8 weeks.

Results

The relative isolation efficiency of TCL and regular mycobacteriology media is shown in table 2.

Table 2.—Comparative isolation efficiency of media

Number of cases	Regular media	TCL medium
8	+	+
25...........................	−	−
3	+	−
1	−	+

+ = Mycobacteria isolated.
- = No isolation.

Based on these data, the isolation efficiency of TCL medium does not exceed that of regular media.

Discussion

The rationale for this experiment was based on improved culture efficiency of media supplemented with bovine lymph nodes (1)[1] and mycobactin (2).

It should be noted that a disproportionate number of media slants were assayed (eight slants of regular media and two slants of TCL medium). This could have introduced a bias favoring regular media but does not alter the fact that TCL medium failed to support growth in three instances when mycobacteria were isolated on regular medium. No record was kept of the number of colonies on each slant.

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[1] Numbers in parentheses refer to References at the end of this report.

In the single instance of isolation on TCL medium only, the culture was identified as *M. avium* serotype 1. Because *M. avium* serotype 2 was used to infect the chickens (for infected livers) the possibility of inoculum surviving the heat treatment was eliminated.

A partisan approach to evaluating the TCL medium would be to consider the value of a single isolation of *M. bovis* which could in some instances lead to the discovery of many tuberculous cattle, their depopulation and ultimate eradication of a source of infection. The animal industry could thereby benefit significantly from the supplemented medium. Conversely, one must consider the extensive procedures involved in preparing TCL medium and the inability to standardize the liver infusion media from batch to batch.

It is the opinion of the project personnel that the advantages of TCL medium do not warrant the added labor and expense involved in its use.

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COMPARISON OF SIX METHODS FOR ISOLATING MYCOBACTERIA FROM SWINE LYMPH NODES. Thoen, C. O., Richards, W. D. and Jarnigan, J. L. Applied Microbiology, Vol. 27, No. 3, Mar. 1974, pp. 448-451. (Abstract of Published Report)

Certain routine mycobacteriological examination methods require elaborate and expensive equipment and are laborious. An increased interest in obtaining rapid diagnostic methods for isolating mycobacteria from animal tissues has resulted from recent changes in Meat and Poultry Inspection Program regulations for handling swine carcasses suspected of being tuberculous. In this report, six different laboratory methods were compared for isolating mycobacteria from tuberculous swine lymph nodes identified by federal meat inspectors at an abattoir in Nebraska.

Tuberculous lymph nodes from each of 48 swine as identified by Federal meat inspectors were processed by each of the methods. Treated tissue suspensions were inoculated onto each of eight media which were observed at 7-day intervals for 9 weeks. There were no statistically significant differences between the number of *Mycobacterium avium* complex bacteria isolated by each of the six methods. Rapid tissue preparation methods involving treatment with 2 percent sodium hydroxide or treatment with 0.2 percent zephiran required only one-third to one-fourth the processing time as a standard method. There were small differences in the amount of contamination among the six methods, but no detectable differences in the time of first appearance of *M. avium* complex colonies.

It is apparent from the data obtained in this study that a substantial financial savings can be realized by using one of the rapid techniques. Furthermore, it should be emphasized that the rapid methods described may be implemented in a diagnostic laboratory without the addition of expensive equipment.

DEVELOPMENT OF A RAPID SEROAGGLUTINATION PROCEDURE FOR THE IDENTIFICATION OF MYCOBACTERIUM BOVIS FROM SWINE AND CATTLE. Jarnagin, J. L. (Project Report)

Summary

Twenty-eight strains of *M. bovis*, 10 strains of *M. avium* and 7 strains of *M. tuberculosis* were tested using a seroagglutination technique. Good correlation was found with *M. bovis* strains but much cross agglutination was observed with *M. avium* strains. The test procedure may be of value in differentiating *M. tuberculosis* strains from *M. bovis* as no cross agglutination was observed between these organisms.

Introduction

The seroagglutination test as proposed by Schaefer (1)[1] has been widely used for differentiating between serotypes of *M. avium*. Seroagglutination procedures for differentiating *M. bovis* and *M. tuberculosis* have not been developed; this may in part be due to the inability to produce smooth cell suspensions with these organisms.

The purpose of this study was to determine if (1) smooth cellular suspensions of *M. bovis* could be produced and used in a seroagglutination procedure and (2) if serologic differences could be detected between strains of *M. bovis*. Information on strain differentiation could have epidemiologic significance.

Materials and Methods

M. bovis strains were isolated from tissues of tuberculous cattle located in four herds in different States. The *M. avium* isolates were strains used previously for antiserum production. Antisera were prepared by intravenous injection of phenol-killed suspensions of *M. bovis* organisms into rabbits following the protocol of Schaefer (1). The antisera were titrated using the homologous strains of *M. bovis* with the titers ranging from 1/40 to 1/80.

Homologous antigens were prepared by inoculating 0.1 ml of a 1- to 2-week-old Dubos Albumin Broth culture of *M. bovis* into 8 ml of Middlebrook's 7H9 broth with added DOAC[2] and 0.1 percent Tween 80 contained in 30 ml plastic tissue culture flasks. The cultures were incubated in a horizontal position for 7 days.

The cell suspension was harvested by transferring the culture to a 20 x 125 mm screw cap test tube and centrifuging at 2640 RCF for 20 minutes. The supernatent fluid was discarded and the cell pellet resuspended in 8 ml of Middlebrook's 7H9 broth without added enrichments or Tween 80. The cells were killed by autoclaving for 15 minutes at 121°C. The autoclaved suspension was allowed to remain overnight at 20°C to permit heavy particulates to sediment. The antigen suspension used in tests was prepared by standardizing to 0.46 optical density at 525 nm in a Bausch and Lomb Spectronic 20 spectrophotometer.

Twenty cultures of *M. bovis*, 2 cultures each of *M. avium* serotypes 1, 2, and 3, 1 culture each of *M. avium* serotypes 4, 8, 9 and 16, and 7 cultures of *M. tuberculosis* were selected for the study. The *M. bovis* strains were selected from known infected herds in various geographical locations.

The seroagglutination test procedure as described by Schaefer (1) was used. Antisera against each of the four *M. bovis* strains and one antiserum each from *M. avium* serotypes 1, 2, 3, 4, 8, 9 and 16 were used.

Results

An analysis of the results (table 1) revealed that the four *M. bovis* antisera were consistent in agglutination with strains of *M. bovis* but cross agglutinated with *M. avium* serotypes 1, 2, and 3. Little cross-agglutination occurred with serotypes 4, 8, 9 and 16. There was also some cross agglutination with antisera of *M. avium* serotypes 3, 5 and 9 and *M. bovis* antigens. Attempts to absorb out the cross reacting agglutinins from the *M. bovis* antisera were not successful.

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[1] Number in parentheses refers to References at the end of this report.
[2] DOAC—Dubos Oleic Albumin Complex—Difco Laboratories, Detroit, MI Catalog #0375-63-1—20 ml per 180 ml of Middlebrooks 7H9 broth base - Difco #0713-01.

Table 1.—Results of M. bovis seroagglutination test

[No. strains reacting—18 hrs. (two tubes)]

Antigen	No. strains tested	Antiserum										
		M. bovis strains				M. avium serotypes						
		1286	471	3046	878	1	2	3	4	8	9	16
M. bovis............	28	25	28	28	25	0	0	5	0	4	5	0
M. avium 1	2	2	2	2	2	1	0	0	0	0	0	1
M. avium 2	2	2	1	1	2	0	2	0	0	0	0	0
M. avium 3	1	1	1	1	1	0	0	1	0	0	0	0
M. avium 4	2	0	1	0	0	0	0	0	1	0	0	0
M. avium 8	1	0	0	0	1	0	0	0	0	1	0	0
M. avium 9	1	0	0	0	0	0	0	0	0	0	1	0
M. avium 16	1	0	0	0	0	0	0	0	0	0	0	1
M. tuberculosis	7	0	0	0	0	0	0	0	0	0	0	0

None of the seven *M. tuberculosis* strains tested cross-reacted with the *M. bovis* antisera. Good agglutination reactions were observed on the 28 strains of *M. bovis* tested with the exception of 2 strains that did not react with *M. bovis* antiserum 878, and 3 strains that did not react with *M. bovis* antiserum 1286.

Discussion

Stable suspensions of heat killed *M. bovis* cells suitable for sero-agglutination examination were produced. The results indicated the sero-agglutination test procedure may be useful in differentiating *M. tuberculosis* from *M. bovis*. Additional strains should be tested to establish statistical validity.

It is interesting and also quite surprising that *M. bovis* appears to be more closely related serologically to *M. avium* than *M. tuberculosis* in view of the amount of nonabsorbable cross reactions to *M. avium* and absence of cross reactivity to *M. tuberculosis*. This observation is in contrast to drug susceptibility characteristics in which *M. bovis* strains are more similar to *M. tuberculosis*.

It was interesting to observe that the strains which failed to react on serologic tests were *M. bovis* cultures from herds in Florida and Puerto Rico, which were observed to have occasional aberrant biochemical characteristics. As serologic differences between *M. bovis* have not been investigated, one may speculate that the failure to show strain variation in serologic reactions is probably due to the *M. bovis* strains selected for antiserum production being of the same or similar agglutination type.

It is conceivable that other *M. bovis* strains selected from cultures with characteristically different biochemical properties might have been more successful as antiserum candidates. Since the onset of this project, some of these strains have been found in routine diagnostic cases and should be investigated.

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DIAGNOSIS OF MYCOBACTERIUM BOVIS AND MYCOBACTERIUM PARATUBERCULOSIS INFECTIONS IN CATTLE BY IN VITRO LYMPHOCYTE IMMUNOSTIMULATION. Alhaji, Idrisu, Johnson, D. W., Muscoplat, C. C., Thoen, C. O. Am. J. Vet. Res., Vol. 35, No. 5, May 1974, pp. 725-727. *(Abstract of Published Report)*

Recent reports revealed that certain types of virus infection could be distinguished by *in vitro* lymphocyte stimulation tests. The purpose of this investigation was to explore the potential use of lymphocyte stimulation in the diagnosis of tuberculosis and Johne's disease.

Peripheral blood lymphocytes from one calf artificially infected with *Mycobacterium bovis* and one cow with naturally occurring infection of *Mycobacterium paratuberculosis* were incubated *in vitro* with purified protein derivatives (PPD) of *M. bovis*, *M. paratuberculosis*, *M. avium*, and *M. tuberculosis* and the degree of lymphocyte stimulation by the PPD was determined according to the incorporation of $[^3H]$-thymidine (^3HdT) in deoxyribonucleic acid (DNA). Peripheral blood lymphocytes from infected cattle were stimulated as much as fifteenfold by the homologous PPD prepared from the mycobacterial species causing infection, whereas heterologous PPD induced little or no stimulation. Lymphocytes from control cattle were unaffected.

The information presented in this report suggests that *in vitro* lymphocyte stimulation test may be a practical and reliable test for diagnosis of bovine tuberculosis and Johne's disease.

MACROPHAGE-DEPENDENT LYMPHOCYTE IMMUNOSTIMULATION IN CATTLE INFECTED WITH MYCO-BACTERIUM BOVIS AND WITH MYCOBACTERIUM AVIUM. Muscoplat, C. C., Thoen, C. O., Chen, A. W., and Johnson, D. W. Infection and Immunity, Vol. 10, No. 3, Sept. 1974, pp. 675-677. (Abstract of Published Report)

Several investigators, using lymphocytes purified from peripheral blood or from lymph nodes, have shown that antigen-mediated lymphocyte immunostimulation is dependent upon the presence of macrophages in the cultures. Many of these studies were performed with animals sensitized with killed mycobacteria, or by using complete Freund adjuvant. No definitive information is available on the role of macrophages in cell-mediated responses of cattle infected with virulent mycobacteria.

In this investigation cultures of bovine peripheral blood leukocytes were prepared from calves inoculated with *Mycobacterium avium* and with *M. avium*. Suitable lymphocyte stimulation was obtained with specific and nonspecific mycobacterial mitogens. Leukocyte cultures depleted of adherent, macrophage-type cells did not respond to stimulation with purified protein derivative, although they were responsive to phytohemagglutinin.

The mechanism of antigen handling by macrophages is poorly understood. However, the results reported herein demonstrate clearly that macrophages perform an essential role in the induction of lymphocyte proliferation in tuberculous cattle.

MYCOBACTERIAL CORD FACTOR. Jarnagin, J. L. (Literature Review)

Microscopic observations of pathogenic mycobacteria, particularly *M. tuberculosis* or *M. bovis* will reveal that the organisms tend to adhere to one another in a parallel fashion and form long stringlike "cords" or "skeins." (figure 1) This phenomenon is caused by cord factor and is most pronounced in virulent strains of mycobacteria.

The purpose of this paper is to (1) describe the chemistry of cord factor, (2) propose a possible biosynthetic pathway and its regulation, and (3) discuss possible modes of action of cord factor upon host cells.

Although cord factor has been synthesized *(8)*[1], no biosynthetic pathway has yet been determined. Despite the lack of an absolute mechanism for the production of cord factor in the mycobacterial cell, the moieties that compose cord factor have been investigated *(7, 9, 12)* and biosynthetic pathways for these components have been proposed.

Noll et. al. in 1956 *(14)* determined cord factor to be the dimycolate ester of trehalose whose chemical formula was determined to be $C_{186}H_{366}O_{17}$.

They found that the mycolic acid moieties were esterified with the two primary hydroxyl groups of trehalose. The mycolic acid components are bound to the number six carbon atoms of trehalose and thus become a 6, 6' dimycolate ester.

The problem lies in the mechanism of condensation of mycolic acid and trehalose to form cord factor. A possible pathway for the biosynthesis of cord factor is proposed utilizing information gained from bacterial cell investigations and synthesis studies of cord factor.

In studying lipid extracts of whole bacilli, cord factor was found to reside primarily in wax fraction C *(8)*.

Pure cord factor was isolated by Noll and Bloch *(13)* as a colorless wax melting at 40° C. In addition, cord factor was found to consist of 85 percent mycolic acid with the remainder being a non-reducing glycoside which upon analysis proved to be glucose. Mycolic acids were discovered by Lesuk and Anderson *(11)* in 1938 in mycobacteria and probably play a key role in the biosynthesis of cord factor.

Since the mycolic acids differ from one strain of mycobacteria to another, several cord factors exist depending on the mycolic acids each strain contains. Lederer *(9)* found that the cord factors of *M. phlei* and *M. smegmatis* have mycolic acids with the general formula of $C_{84}H_{164}O_6$.

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[1]Numbers in parentheses refer to References at the end of this report.

The biosynthesis of the mycolic acid component has been studied by Lederer *(7, 9)*. He found mycolic acids to be long chain fatty acids containing 84 to 88 C atoms. They characteristically had a long aliphatic side chain on the alpha C atom and a OH group on the beta C atom.

Four of these fatty acids condense to form a complete molecule of mycolic acid. This mechanism is illustrated in figure 1. The synthesis of these fatty acids is not completely resolved but may be similar to the acetate-malonate mechanism found in mammalian cells.

The disaccharide trehalose is found in mycobacteria in significant amounts and is most likely synthesized there from glucose. The reaction utilizes a nucleoside diphosphate glucose such as uridine diphosphate glucose (UDPG) or guanidine diphosphate glucose (GDPG) which reacts with glucose-6-PO_4 to form trehalose-6-PO_4. The reaction occurs as follows: *(12)*

$$\text{UDP glucose + glucose 6-PO}_4 \xrightarrow{\text{trehalose synthetase}} \text{trehalose-6-PO}_4 + \text{UDP}.$$
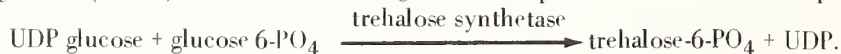
How trehalose is esterified to mycolic acid is only speculative but formation of other glycolipids in other bacterial systems may provide a clue.

Burger *(2)* and others studied the enzymatic synthesis of a rhamnose glycolipid in *Pseudomonas aerouginosa.*

The glycolipid studied was 1-rhamnosyl-1 rhamnosyl-B-hydroxy decanoyl-B-hydroxy decanoate. It was found that thymidine diphosphate-1-rhamnose and B-hydroxyl decanoate react directly to form the product.

Lennarz *(10)* et. al. studying the formation of mannolipids in *Micrococcus lysodeikticus* found that the formation of mannosyl-mannosyl diglyceride occurred in a stepwise manner by addition of the mannose moieties one at a time to the lipid component of the molecule.

In both of these cases the sugar moiety was added directly to the lipid component utilizing a nucleoside sugar and most probably an esterase to catalyze the reaction. One may speculate that cord factor may be produced in a similar way by direct esterification of trehalose with mycolic acid. The described studies in other bacterial systems and investigations into the synthesis of cord factor *(8)* by direct esterification add credibility to this hypothesis.

The regulation of cord factor synthesis may be approached as a function of synthesis of its two compenents, mycolic acid and trehalose. Similar to the elucidation of the biosynthetic pathway the work performed on other bacterial systems may be helpful in determining the mode of regulation in the mycobacteria.

Henderson and MacNeill *(4)* studied the control of fatty acid synthesis in *Lactobacillus plantarum.*

Using C *(14)* acetate in radioactive tracer studies they found that long chain unsaturated fatty acids had a negative feedback control in fatty acid synthesis. Their results indicated that one site of inhibition is the acetyl CoA carboxylase enzyme.

Birnbaum *(1)* found that biotin will repress fatty acid biosynthesis. Further studies by this investigator revealed that biotin was not the true repressor but acts upon the unsaturated fatty acids which are the true feedback repressors.

The regulation of trehalose in the mycobacteria has only recently been studied. Nikaido *(12)* et. al. found two fractions that would catalyze the synthesis of trehalose. Fraction A catalyzed synthesis from GDP-glucose while a combination of fraction A + fraction B utilized UDP glucose. It was found that crude lactalbumin could stubstitute for fraction B. The active component in the lactalbumin was RNA which would be degraded by RNAase. This opened the possibility of natural RNAase in the bacterial cell as regulators by acting on fraction B.

Lapp and Elbain *(6)* found that in addition to GDP glucose and UDP glucose, glucose diphosphates of adenine, cytidine and thymidine would also work with trehalose synthetase to produce trehalose in mycobacteria.

Their investigations suggested the possibility of feedback inhibition as the end product of the reaction, trehalose phosphate, would inhibit ADP glucose and GDP glucose but did not affect TDP glucose.

The toxic action of cord factor upon host cells infected with virulent mycobacteria has been well studied. Investigations by Panos and Ajl *(15)* revealed that cord factor may be involved in inhibiting succinic dehydrogenase as removal of cord factor restored the enzyme to previous activity.

Kato and Fukushi *(5)* showed that cord factor when introduced into mouse liver cells, induced swelling and disintegration of mitochondrial membranes. These investigators also found evidence of biochemical lesions in mitochondrial sites involving respiration and oxidative phosphorylation. Fukuyama *(3)* et. al. recently postulated that cord factor induces disintegration of the endoplasmic reticulum and allows the ribosomes to detach resulting in repression of host protein synthesis.
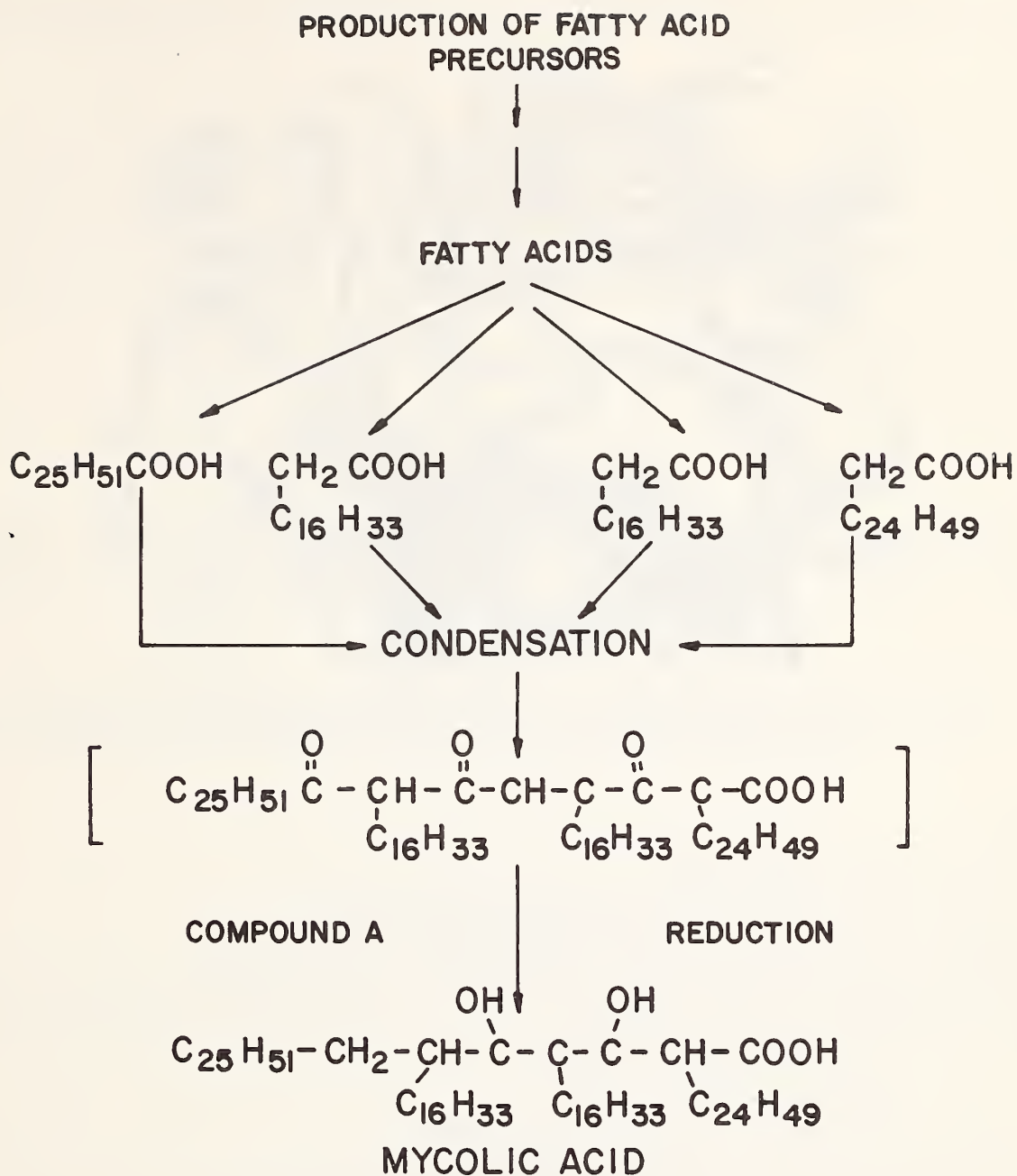
Figure 1.—Proposed biosynthetic pathway for mycolic acid. Four fatty acids condense to form an intermediate (Compound A) which is further reduced to mycolic acid.
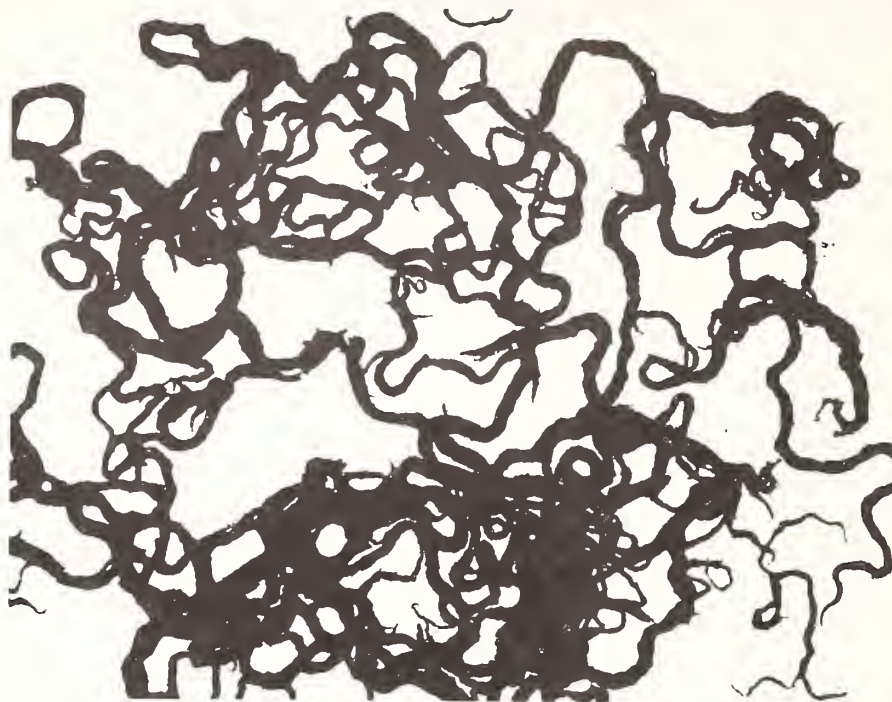
Figure 2.—Example of "cording" in a smear of an *M. tuberculosis* culture stained with carbol fuchsin. × 400.

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PHAGE SUSCEPTIBILITIES OF MYCOBACTERIUM BOVIS. Richards, W. D. Proceedings of the 7th International Symposium on Phage Typing of the Mycobacteria - Pisa, Italy, September, 1974. (Abstract of Published Report)

Susceptibility to mycobacteriophages was investigated as a possible means of subspecies identification of *Mycobacterium bovis*. Of 29 phages obtained from other investigations, 10 were selected for the evaluation. Approximately 200 *M. bovis* isolates were challenged with the 10 phages and all tests were made in duplicate.

Reproducibility of results was unacceptable until corrective procedural changes were made. Although reproducibility was improved, phage susceptibility patterns were not applicable for differentiating between *M. bovis* cultures isolated from cattle in different herds. Marked differences were seen between susceptibility patterns of *M. bovis* isolated from cattle, *M. bovis* isolated from a tapir and an *M. bovis* tuberculin stock culture.

NEWCASTLE DISEASE

VIRUCIDAL ACTIVITY OF COMMERCIAL DISINFECTANTS AGAINST VELOGENIC VISCEROTROPIC NEWCASTLE DISEASE VIRUS. Wright, H. S. Avian Diseases, Vol. 18, 1974, pp. 526-530. (Abstract of Published Report)

Five commercial disinfectants were tested for effectiveness against velogenic viscerotropic Newcastle disease virus (isolant AAF/2). Virus suspension containing $10^{8.0}$ 50 percent egg-lethal doses per 0.1 ml. was treated with the disinfectants. All were effective virucides at the manufacturers' recommended concentrations.

PASTEURELLA

IMMUNE RESPONSE EVALUATION OF CATTLE, SHEEP, AND SWINE TO PASTEURELLA MULTOCIDA. Vogel, J. H. and Bairey, M. H. (Project Report)

Introduction

Early in 1971 the leaders in Animal and Plant Health Inspection Service (APHIS) determined that the efficacy of the mixed bacterins must be demonstrated or the products removed from the market. APHIS, through the biologics director, informed the biologics industry that they must prove this efficacy. Biologics Laboratory personnel were instructed to begin developmental studies to establish potency assay methods for the bacterin products.

Materials, Methods and Results

The method chosen was to give priority to mixed bacterin aerobic bacterial components which are also found in polyvalent vaccines and bacterins. Selection was based on the economical importance from a disease standpoint and also the number of doses of bacterin containing this organism that was produced and marketed.

Pasteurella multocida, an important pathogen, was selected.

After selection, a project was designed to evaluate the pathogenicity and immunogenicity of *P. multocida* in host animals other than avian.

The approach selected to obtain the most meaningful evaluation for efficacy was to demonstrate protection against exposure of the disease causing organism in the host animal and to correlate these results with a laboratory animal protection test. This would be done by vaccinating the species of animal with a bacterin prepared to cause an immune response and then exposing the animals to the virulent organism.

In making infectivity or challenge trials in cattle, sheep, and swine to determine which could be infected consistently and produce reproducible clinical signs, nine experiments were made using a total of 66 pigs, 69 calves and 97 lambs. With a few exceptions, the lambs and calves were from local Iowa farms. Some pigs were hysterectomy derived-colostrum deprived (HDCD) and some were specific-pathogenic free (SPF) second generation.

Because of the complexity of the infection process with *Pasteurella multocida* under field conditions, an inoculation route which would give reproducible results was needed. Intranasal, intratracheal, intramuscular, intravenous (IV), intraperitoneal, and oral routes were tried. Other factors considered essential for a reproducible challenge were to be able to quantitate the dose size and approximate the number of live organisms each animal received to produce certain clinical signs.

It was concluded that a consistent reproducible infection of acute pasteurellosis could not be experimentally produced in calves using our methods of infectivity.

It was also demonstrated that sheep were infected IV with strains homologous in origin, heterologous in origin, and with strains that differ in serotype.

The consistent signs of infection that were observed in addition to death were temperature elevation, depression, anorexia, rapid respiration, abdominal pain, weakness, recumbency, lameness, and swelling primarily of the hock and stifle joints. Swine also appeared to be a satisfactory animal for protection studies when challenge was inoculated IV.

The consistent clinical signs of infection were similar to those found in the lambs. Lateral recumbency referred to as the "downer syndrome" appears more in swine than in lambs. The affinity *Pasteurella multocida* has for the joint tissues and synovial fluid causing severe pain, swelling, lameness, and pathological lesions appear to be the cause of the "downer syndrome".

Bacterins containing 67 to 6.7 billion organisms per dose were given subcutaneously to pigs. The purpose of the wide range in count was to establish a satisfactory and unsatisfactory bacterin. The animals were vaccinated day 0 and revaccinated day 14 followed by an IV challenge inoculum 14 days after the second vaccination with 120 million viable organisms per pound of body weight.

Protection against death was produced by all bacterin concentrations with only one death out of 12 vaccinated animals tested. However, a quantitative differentiation was observed with 100 percent protection against infection produced when 33.5 billion organisms/dose were used in the bacterin, 50 percent protection using one-half the number of organisms and 0 percent protection with one-fourth the organisms.

In preliminary challenge studies in lambs, it was concluded that lambs could be infected with *Pasteurella multocida*. Vaccination-challenge studies similar to swine experiments have not provided the same information. However, Pasteurella bacterins containing 25 billion organisms per dose have provided protection against death but not against infection (primarily lameness) using a homologous challenge.

The second part of the developmental work was to design a laboratory animal assay method for evaluating bacterins which would correlate with the protection in the host animal. In surveying the literature, a mouse protection test using the Ose-Muenster (1)[1] method of calculating the results was selected. This procedure was reproducible and had the least amount of variables.

The method is a vaccination-challenge test scheme in mice. Groups of 10 mice each are vaccinated day 0 and revaccinated day 14, then challenged 10 days later with tenfold dilutions of virulent *P. multocida* culture. Unvaccinated control mice are also inoculated with tenfold dilutions using groups of 10 mice each. The 50 percent endpoint of mortality (LD_{50}) is calculated by the method of Reed and Muench (2) for both the vaccinated mice and the unvaccinated controls. The LD_{50} of the vaccinated groups is subtracted from the LD_{50} of the unvaccinated controls to give the number of logs protection.

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[1] Numbers in parentheses refer to References at the end of this report.

It appears as if bacterins giving three or more logs protection in mice will give 100 percent protection in swine, those giving approximately two logs protection in mice give 50 percent protection in swine and those giving less than two logs will not protect swine against infection by our test procedure.

Work on the efficacy of the *Pasteurella multocida* component of mixed bacterins and how to measure it are in process. Experiments being conducted and those proposed in the future should provide answers to questions that are of concern to practitioners and livestock owners as well as the biological industry.

Discussion and Conclusion

Conclusions thus far are: (1) in the lambs and calves, there appears to be little difference regarding the response to intravenous challenge in animals in which Pasteurella was isolated from nasal swabs and those that were negative. Also, there was a consistent response to challenge in swine regardless of whether they were HDCD or SPF second generation, (2) swine and sheep can be experimentally infected with *P. multocida*, (3) swine can be protected against a severe challenge inoculation of *P. multocida* with an experimental bacterin, (4) there is a quantitative relationship between protection in the animal and the amount of antigen in the bacterin, and (5) double vaccination at 21-day intervals or longer may be necessary.

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POISONING

ARSENIC POISONING IN WILD DEER. Nelson, H. A. (Case Report)

Liver tissue was submitted to VSL, Toxicology Section, from a deer found moribund in a cotton field. A farmer cultivating the field accidentally ran over and killed one deer and observed another lying down unable to rise. It died 2 or 3 hours later. The animal was in fairly good flesh and pregnant. The cotton field in which the deer were located had been sprayed about 2 weeks previously with either monosodium methanearsonate (MSMA) or disodium methanearsonate (DSMA). These compounds are herbicides containing organic arsenic as the active ingredient. Application rate for MSMA is two pounds actual per acre. This rate of application is considered hazardous to cattle (2)[1]. Application rate for DSMA is 3 pounds actual per acre. This rate of application is also considered hazardous to cattle and sheep (2).

Liver tissue submitted was analyzed for arsenic residue. The level of arsenic present was 15.75 parts per million (wet weight), which is consistent with a diagnosis of arsenic poisoning (1).

This case demonstrates potential hazards of organic arsenical herbicides for wildlife. Animals such as deer have no control over their environment and are at the mercy of modern agricultural practices. When organic arsenic herbicides are used in areas adjacent to those in which deer roam, the danger of poisoning is present. In addition to initial poisoning from eating contaminated vegetation, there is the danger of arsenic remaining in the soil, creating a potential for wildlife exposure over an extended period of time.

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[1] Numbers in parentheses refer to References at the end of this report.

Consideration should be given to immediate- and long-term effects on game animals from arsenic containing herbicides and other persistent type chemicals. A survey of tissues (particularly liver) from animals that die or are harvested during the hunting season would indicate the extent of exposure.

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LEAD POISONING. Nelson, H. A. and Miller, S.[1] (Case Report)

Lead has been and continues to be one of the major causative agents in poisoning of domestic animals. Sources range from discarded batteries to paint, grease, roofing materials and industrial pollution.

An awareness of the toxicity of lead and the variety of sources is important to the livestock producer and the practicing veterinarian.

In a case submitted to the VSL for toxicological analysis, six yearling calves were affected with what was suspected to be lead poisoning. These calves obtained their water from a can formerly used for paint. Three calves were found dead and three calves were exhibiting signs indicative of lead poisoning. Signs commonly exhibited are depression, anorexia, abdominal distress, twitching of ears, bobbing of head, muscular tremors, ataxia and pushing against objects. Excitement and convulsions may also occur.

In a survey conducted by Leary *et al* (2),[2] 90 percent of cattle affected with lead poisoning exhibited signs of central nervous system involvement and 60 percent exhibited signs of gastrointestinal involvement. Paint was the most common source and was involved in 29 percent of the cases.

Chemical analysis of a sample of paint residue from the watering can and a kidney submitted from one dead calf was conducted using an atomic absorption spectrophotometer for detection and quantitation of lead. The kidney was found to contain 85 ppm lead and the paint sample contained 35 percent lead (350.000 ppm).

Levels of 10 ppm or more in the kidney are suggestive of lead poisoning and values of 25ppm or more in the kidney cortex are considered diagnostic. (1, 2) In 39 cases reported by Leary *et al* (2) the mean kidney lead level was 63.9 ppm, and in a survey by Hatch and Funnell (1) the average kidney level was 137 ppm. The 85 ppm lead found in this case was well within the range considered to be diagnostic of lead poisoning. The paint residue in the watering pail demonstrates the source and also serves to emphasize the high level of lead which may be in paint. Livestock owners should be made aware that these containers should not be used as feeding or watering utensils for livestock.

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[1] Director, Regional Animal Health Laboratory, Moorefield, W. Va.
[2] Numbers in parentheses refer to References at the end of this report.

PSEUDORABIES IN THE UNITED STATES. Stewart, W. C. (Literature Review)[1]

Summary

Pseudorabies (PR), formerly known as "mad itch", apparently occurred in cattle in the Midwest more than 125 years ago. Although a relationship between cattle and swine was suspected, it wasn't until after the epizootiological role of swine was discovered in the early 1930's that the disease was recognized in this species. Initially, PR was recognized in neonatal pigs and was characterized by signs of a central nervous system (CNS) derangement. Pseudorabies has been a disease principally of suckling pigs, although several epizootics, noted particularly for their pathogenicity in breeding and feeding swine, have occurred in herds with continuous farrowing and feeding operations.

Pseudorabies occurs sporadically, but it has a wide distribution and has probably occurred at one time or another in all States having a large population of swine. While the husbandry practices of running cattle and swine together have diminished over the years, PR still occasionally affects other highly susceptible species having contact with diseased swine. Limited serologic surveys indicate that the incidence of PR is presently at a low level. This reduction in incidence is attributed to general control measures instituted in the national hog cholera eradication program.

Swine are believed to be the principal if not the sole reservoir of PR. A low percentage of infected swine may remain carriers and spread the virus to susceptible contacts. Maintenance of the virus for several months in the persistently infected fetuses of pregnant sows is also a distinct possibility. There is some evidence to suggest that stress may play a role in causing an exacerbation of the disease in carrier swine. The disease is sustained through the extensive movements of swine in commerce and perhaps the feeding of pork scraps in raw or improperly cooked garbage.

History of the Disease

According to Hanson (4),[2] epizootics of PR first appeared in cattle in the Midwest more than 125 years ago. This conclusion was based on published reports of that era describing a rubbing disorder, or "mad itch", which appeared to agree in all respects with the signs of PR: short incubation period, pruritus, fatal course, susceptibility of cattle and dogs, and the association between cattle and swine as a factor of epizootiological significance. While greater restrictions on the range of swine may have been responsible for an apparent decline in the incidence of "mad itch" after 1860, isolated epizootics continued to appear.

However long the disease may have been present, it wasn't until 1931 that Shope (12) demonstrated that "mad itch", occurring in cattle in Iowa, was caused by a filtrable virus which was immunologically identical with Aujeszky's (1) Hungarian strain of PR virus. Later, Shope (13) concluded from experiments on the epizootiology of PR that swine were the source of infection for cattle. Transfer of the virus was thought to occur from the noses of swine through the abraded skin of cattle. Whereas the virus transmitted readily among swine, the disease was generally mild and went unrecognized.

Shope (14) concluded from the results of serological studies that PR was a highly prevalent, unrecognized disease in Midwestern swine. In 21 of 23 lots of pooled sera, 5 to 50 percent of the animals had antibody titers indicating a previous encounter with PR virus. In like manner, 13 of 15 swine bled at a biological house and 10 swine picked at random from a local abbatoir had antibodies against PR virus. In contrast to the relatively large number of Midwestern swine with PR antibodies, there was complete absence of antibodies in swine tested in the State of New Jersey. Although the discovery by Shope provided a clearer understanding of the epizootiology of PR, it added another dimension to the disease, namely, its involvement as a clinical entity in swine.

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[1] Published in "Les Cahiers de Medicine Veterinaire" in French.
[2] Numbers in parentheses refer to References at the end of this report.

Evolution of the Disease

Having discovered the epizootiological role of swine in the transmission of PR to cattle, it is not surprising that clinical disease in swine following natural infection was soon recognized. In 1943, Ray *(8)* reported on a disease in neonatal pigs which he attributed to PR. The pigs had signs of incoordination and excitability which progressed to paralysis, prostration, and death. Mortality in 190 pigs was approximately 45 percent. When inoculated into rabbits and mice the action of the virus was identical with strains of the virus isolated from cattle.

An unusual epizootic of PR in 3 swine herds within a 5-mile radius in Indiana was described by Saunders *et al. (9)* in 1963. In two herds signs of the disease first were observed in gilts and 3.5 month old feeders and later appeared in suckling pigs. Signs of the disease were anorexia, vomition, constipation, abortions, and CNS derangement. The strain of virus was unusually pathogenic for swine; however, an age resistance factor was clearly present. Mortality was about 55 percent in newborn pigs and declined to approximately 3 percent in market swine. Mortality was rare in sows.

In a subsequent report, Saunders and Gustafson *(10)* gave an account of the cases of PR confirmed in Indiana from January 1962 to October 1964. Of 13 cases, 3 involved primarily cattle, but in each instance there was contact with swine. Ten cases involved primarily swine with associated deaths in cattle, dogs, and sheep. The disease in swine was notable for its virulence. Further substantiation of the presence of PR strains causing mortality in older swine was reported by Gustafson *(3)*. In this instance, 60-to 80-pound feeders were assembled at a common location and distributed to four separate premises where PR occurred simultaneously. Overall mortality on the four farms was approximately 9 percent or 200 to 2,500, 38 to 300, 38 to 250, and 47 to 500.

Howarth and DePaoli *(5)* described an enzootic of PR occurring over a 3-year period on a large California hog ranch where cooked garbage was fed to 6,000 swine in a continuous feeding operation. Four hundred feeders were added to the ranch every 2 weeks. In all acutely ill pigs there was derangement of the CNS and a significant degree of blindness. The course of the acute disease averaged 3 days after which a pig was either dead or essentially recovered. The disease was noteworthy because of the high mortality in pigs weighing 80 to 140 pounds. During the 3-year period, 1,687 deaths were attributed to PR.

Although several cases of PR in feeders have been reported in recent years, the disease appears to involve primarily young pigs. In a report by Stewart *et al.(15)*, the age of the pigs principally affected in 38 epizootics of PR occurring over a period of several years was determined. Pigs up to and including 6 weeks of age were considered sucklings and pigs 7 weeks and older, weanlings. Of the 38 cases, sucklings were affected in 28 and weanlings in 10. Occasionally, the disease appeared first in weanlings with low mortality and later in sucklings with high mortality.

Geographic Distribution

Governmental regulations do not require the reporting of epizootics of PR so an account of its geographic distribution would be incomplete. However, with evidence of the disease having been present in the United States for more than 125 years and knowledge of the extensive swine movements occurring in today's commerce, it is logical to assume that PR has occurred in all States with substantial swine populations.

An indication of the widespread distribution of PR is manifested in a report by Stewart *et al.(15)* Sixty-seven cases from 11 States were confirmed as PR at the Veterinary Services Laboratory from 1966 to 1972. The number of States represented in each geographical region were Midwest (6), South (4), and West (1). The greatest number of cases (41) yielding PR isolates were received from the State of Indiana. Subsequent to the report, PR virus has been isolated from specimens received from Texas, a State located in the southwestern part of the United States (fig. 1).

Pseudorabies has been reported in cattle in Minnesota *(11)* and Wisconsin *(7)* and in dogs *(2)* in Alabama, Florida, Georgia, and Louisiana. According to Gustafson *(3)*, virulent infections of PR have occurred in swine in the States of Massachusetts, Pennsylvania, Michigan, Georgia, Florida, Missouri, and California.

Epizootiological Characteristics

Pseudorabies as seen in the United States is principally a disease of suckling and weanling pigs characterized by signs of CNS disturbance and macroscopic lesions of a septicemia (8, 15). However, the virus is capable of increased pathogenicity for both immature and mature swine as evidenced by virulent infections occurring in intensive continuous breeding and feeding operations in Indiana (9) and California (5). Occasionally, the disease spreads into populations of other domestic animals having contact with swine such as cattle, sheep, dogs and cats (3, 15). Although these species are susceptible, available evidence suggests that they may be dead-end hosts for the virus.

Considerable attention has been given to the method by which PR is maintained. In the absence of more compelling evidence, swine, the natural host of the virus, are considered to be the reservoir. Apparently, a low percentage of infected swine become latent carriers and shedders of the virus. There is strong evidence that virus is eliminated from the upper respiratory tract of these carriers to susceptible swine. Of perhaps equal importance in dissemination of the virus is the carrier sow. She may carry the virus to termination of pregnancy or shed it abruptly in an abortion. Ample opportunity is presented for the intermixing of these carriers and susceptible contacts as swine are assembled at commercial concentration points for redistribution (3, 9).

Numerous observations support the hypothesis that PR is maintained through its reservoir in swine. In Indiana, four of five epizootics of PR investigated in 1964 occurred in the same county in which five epizootics were encountered in 1962-63. One epizootic in 1964 involved a herd in which the disease had occurred in late 1962 (10). Two infected pigs kept in isolation were reported by Saunders et al. (9) to have exacerbation of clinical signs for 1 month and one pig had a persisting microscopic foci of encephalitis 5 months after the acute phase.

Stress factors may be important in affecting the shedding state or causing an exacerbation of the disease in carrier swine. While investigating an enzootic of PR in a herd over a 3-year period, Howarth and DePaoli (5) observed that in each instance the onset of disease was sudden and was preceded by a drastic change in climatic conditions. Either warm or cold weather precipitated the disease which continued as long as extreme weather persisted. Permanent blindness was a sequela in a small number of pigs that recovered.

Another possible source of the virus is improperly cooked garbage containing pork scraps. A serologic survey disclosed that PR was present on eight of nine garbage feeding ranches in California (6). The most plausible source for one dog contracting PR was garbage (2). The transportation of virus between herds by dogs feeding on infected swine has also been suggested (9).

Control Methods

The sporadic nature of PR has apparently been responsible for the absence of any concerted effort to eradicate or control the disease nationwide. However, general control measures, acquired as fringe benefits of the national hog cholera eradication program, have served as a regulating factor in the incidence of PR. Control measures applying to PR as well as to hog cholera are the establishment of special feeder pig sales with prior inspection of the pigs on the farm of origin, inspection at the markets in all other cases, cleaning and disinfection of the markets between sales, segregation of the feeding and breeding swine within the markets, quarantine and isolation of swine after arriving at the farm of destination, and prohibition or restriction on the feeding of garbage to swine.

When epizootics of PR occur, quarantines by regulatory officials and specific control measures on the farm may be used to check the disease. Relative to the latter, the removal and isolation of sick swine is very important. Since recovered animals can be carriers of the virus, isolation should be continued until the animals can be marketed. Measures should be taken to preclude the intermixing of feeding and breeding swine. Susceptible species such as cats, dogs, cattle, and sheep should be separated from swine. A rat control program and general hygienic and husbandry practices are other measures that should be considered.

Vaccines are not available from commercial firms largely because the small demand for such products makes their production economically unfeasible. The results of using vaccines against a disease with such a low incidence could be one of diminishing returns; that is, the vaccine could contribute to the problem by the establishment of carriers or persistently infected swine which shed the virus (3). Saunders and Gustafson (10), while investigating the protective properties of immune serum in pigs experimentally infected with PR, reported that mortality was higher in the treated as compared to the non-treated group. Thus, it seems the efficacy of immune serum in epizootics of PR is questionable.

Perspectives

As compared to former serologic studies *(14)*, recent surveys in California *(6)* and Indiana *(10)* and the results of serologic tests performed on diagnostic cases at the Veterinary Services Laboratory (unpublished) suggest a low incidence of PR. While it is possible that epizooties of the disease could go unrecognized, the available evidence in cases where PR has been confirmed suggests that this is not a problem *(15)*. Therefore, it appears that the disease has reached a point where further reduction will not occur unless specific control measures are applied.

In the event of the relaxation of general control procedures in the hog cholera eradication program, it is conceivable that an increase in the incidence of the disease could result. With the extensive swine movements occurring today, conditions would be suitable for the evolvement of highly pathogenic strains of the virus *(3,5,9)*. Should this occur, a program embracing specific measures of control would then become necessary.

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STUDY OF DISSEMINATION OF RABIES VACCINE VIRUS IN DOGS AND CATS. Bunn, T. O. and Strating, A. (Project Report)

Summary

Each of the strains used to produce modified live virus rabies vaccines was inoculated intramuscularly or intraneutrally into dogs and cats. Tissue samples were collected at periodic intervals and examined by the fluorescent antibody (FA) technique and suckling mouse inoculation for the presence of vaccine virus. ERA virus was recovered from the sciatic nerve 1 and 3 days after intraneural inoculation.

Introduction

Because of reported false positive results obtained with the FA technique for rabies diagnosis in dogs vaccinated with the Flury low-egg-passage (LEP) strain of rabies vaccine *(8)*,[1] and because this strain has been reported to cause paralysis in young dogs *(7)*; several workers *(2, 5, 9)* have investigated the fate of LEP virus after vaccination. In 1973 the United States Department of Agriculture's standard requirement for modified live virus rabies vaccine was rewritten *(1)* to require a fate of the virus study be conducted by each licensee using a representative sample of the vaccine they produce. This is a report of preliminary work done with each vaccine strain available in the United States.

Materials and Methods

Animals—Thirty-six beagle dogs and 24 cats were divided into 6 and four groups respectively containing 6 animals each. All animals were between 5 months and 1 year of age and were serologically negative for rabies antibody prior to vaccination.

Suckling, less than 7 days old, white Swiss mice were used for virus isolation.

Vaccines—Commercial vaccines produced from the low-egg-passage and high-egg-passage Flury strains and from the ERA strain of rabies virus were used. The virus content of each vaccine was above the minimum titer established in the standard requirement *(1)*.

Experimental Design—Each vaccine was inoculated into one group of animals intramuscularly and into another group intraneurally. The high-egg-passage Flury vaccine and the ERA vaccine were used in both dogs and cats while the low-egg-passage Flury vaccine was restricted to dogs.

For the intramuscular inoculation, the vaccine was reconstituted to the recommended volume and inoculated into the thigh muscles of the left leg. For the intraneural inoculation, the left sciatic nerve was surgically exposed and the vaccine, reconstituted to 1 ml, was injected under the nerve sheath.

One animal from each group was sacrificed on post-inoculation day 1, 3, 6, 9, 14, and 28. Samples of the left sciatic nerve, external iliac lymph node, kidney, and mandibular salivary gland were aseptically removed. Tissue samples were also taken from the liver, mesenteric lymph node, lumbar spinal cord, medulla oblongata, and both hippocampi. Two samples of each tissue were collected; one for the FA test and one for the mouse inoculation test.

Prior to sacrificing each animal, 30 ml of blood was collected, heparinized, and centrifuged at 500 x g for 15 minutes. The buffy coat was removed and stored, along with the tissue samples, at $-70°$ F until tested for the presence of rabies virus.

Blood smears were prepared from the animals that remained on day 1, 3, 6, 9, 14, and 28. The slides were immediately stained by the FA technique.

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[1] Numbers in parentheses refer to References at the end of this report.

Fluorescent Antibody Technique—Tissue specimens were frozen, sectioned, fixed, and stained by the method of Goldwasser and Kissling *(4)* using fluorescein isothiocyanate conjugated anti-rabies globulin of burro origin. Blood smears were stained in the same manner.

Suckling Mouse Inoculation Technique—Tissue samples were ground in a Ten Broeck grinder using distilled water plus 2 percent horse serum and antibiotics as a diluent. At least five suckling mice were inoculated intracerebrally with .02 ml of tissue suspension. The mice were observed for 21 days and the brains of any mice that died after 5 days were checked by the FA technique for specific rabies fluorescence before being considered positive.

Results

All blood smears and tissue sections were negative when examined by the fluorescent antibody technique. All tissue and blood samples were negative when inoculated into suckling mice except for the groups that received ERA vaccine intraneurally. In these groups, the sciatic nerves of the dog sacrificed on day 1 and the cats sacrificed on days 1 and 3 caused death in mice 8 to 10 days post-inoculation. The brains of these mice exhibited typical rabies fluorescence.

Discussion

The negative results obtained with the Flury vaccines agree with previous work done with the low-egg-passage strain *(2,5,9)*. The presence of ERA virus in the sciatic nerve 1 and 3 days after intraneural inoculation may have been due to residual inoculum rather than a multiplication of the injected virus. It should be noted that the lack of fluorescence in the sciatic nerve does not indicate a lack of replication since even with virulent street rabies, no fluorescence can be detected in peripheral nerves until after the virus has reached the spinal cord *(6)*.

The stability of the different strains at $37°$ C. and the number of suckling mouse brain lethal doses of each strain injected need to be established since these factors may have influenced the isolation of one strain over another.

The safety of the vaccine strains is indicated by the absence of virus in all tissues sampled except for the injection site. The absence of virus in the kidneys indicates that there is limited chance for the excretion of vaccine virus via the urine, and subsequent intranasal infection of a more susceptible animal. This has been suggested as a possible method for transmission of street rabies *(3)*.
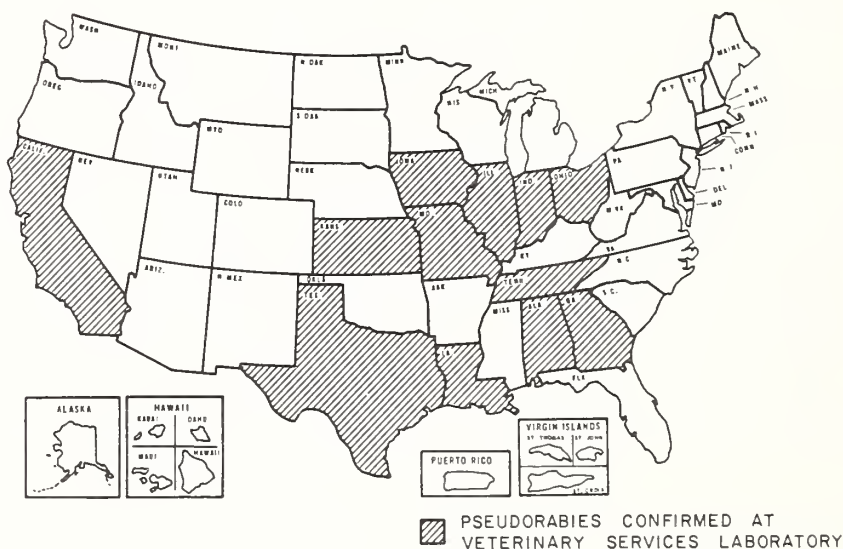
Figure 1

Similar fate of the virus studies are being conducted by all United States Department of Agriculture licensed commercial producers of modified live virus rabies vaccine. It is hoped that the information obtained will add to the knowledge regarding the safety of these products.

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SALMONELLA

IMMUNE RESPONSE TO SALMONELLA TYPHIMURIUM. Bairey, M. H. and Vogel, J. H. (Project Report)

Introduction

Salmonellosis is a widespread disease of domestic animals and birds which has been recognized on all of the continents *(4).*[1]

Cattle of all ages can be infected with *Salmonella typhimurium* but calves between the ages of 2 to 6 weeks seem to be the most susceptible to acute and fatal infections *(2, 4)*. The clinical disease may occur sporadically or as a herd epizootic *(3, 5, 7)*.

The most common clinical signs at the onset are fever, anorexia, and fetid diarrhea. As the disease develops, depression, dehydration, and emaciation are evident. Some cases will develop pneumonia, nervous signs, and joint swellings with an arthritic condition *(2, 4, 8, 1)*.

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[1] Numbers in parentheses refer to References at the end of this report.

Postmortem findings will commonly include some degree of enteritis, enlargement and edema of the mesenteric lymph nodes, and a swollen and edematous gall bladder. Occasionally the lesions of pneumonia or a generalized septicemic condition will be present *(2, 8, 6, 1)*.

The only *S. typhimurium* bacterins that are licensed for commercial use in the United States today are in combination with *S. dublin* and are formalin killed, aluminum hydroxide adjuvanted products. This investigation was designed to determine if these bacterins would provide protection against challenge inoculation of a live virulent culture of *S. typhimurium* which would produce clinical disease in unvaccinated animals. Also, the development of a mouse protection test which would provide a means of evaluating these bacterins on a serial to serial basis was investigated.

Materials, Methods and Results

Mice.—According to the literature, mice would be a desirable laboratory animal to use in *S. typhimurium* infection and protection studies. However, as there is almost no published information on *S. typhimurium* infection in other laboratory animals, the susceptibility of rabbits, guinea pigs, and gerbils as well as mice was evaluated.

The culture used was a stock culture identified as 6352. It was selected because it had the biochemical and antigenic properties of a typical *S. typhimurium* serotype. It was prepared for animal inoculation by incubating in beef infusion broth for 16 hours. This had previously been determined as the optimal growth period for pathogenicity. The culture suspension was standardized to a 43 percent light transmittance 620 nanometers on a Bausch and Lomb Spectronic 70 Spectrophotometer by diluting with Nutrient Broth.

All animal species were inoculated intraperitoneal (IP) using doses of 5 ml in rabbits, 2 ml in guinea pigs, 1 ml in gerbils and 0.1 ml in mice.

Mice were selected as the laboratory animal of choice based on susceptibility and consistency of death pattern.

Calves.—To successfully show protection in calves, it is necessary to be able to consistently infect calves under controlled experimental conditions.

Dairy breed calves of about 6 weeks of age of both sexes could be obtained readily and were used in all experiments. The route and dose of challenge inoculation that would produce distinct and reproducible signs of infection needed to be determined before protection studies could be undertaken.

It was decided that intramuscular (IM) was the optimal challenge route for reproducibility of results. Death and clinical signs both could be used as infection indicators.

Two experimental bacterins with a tenfold difference in antigen concentration and a commercially manufactured bacterin produced protection against the clinical signs of disease.

Bioassay Procedure.—After establishing the efficacy of the reference bacterin and one commercially manufactured bacterin in calves and mice, it was necessary to compare the results of these assays. A bacterin containing 3 billion organisms per ml will protect calves against death and clinical signs. The average mouse protection over three replication was more than six logs.

When the concentration of the bacterin was reduced by one-half, all calves were protected from death against the lethal challenge and two of three were protected from all clinical signs. One trial in mice produced just under six logs protection.

A commercial bacterin protected against this same challenge with all animals vaccinated showing no clinical signs. The average protection of six replications in mice was over five logs.

After establishing that a bacterin produced from a *S. typhimurium* culture would protect against a severe disease produced by challenge inoculations of *S. typhimurium*, it was speculated that bacterins prepared from other serotypes of Salmonellae might protect against this same challenge. Since it had been shown that mouse protection of five logs or better would be indicative of this protection in calves, the mouse bioassay procedure was used to evaluate this theory.

Reference bacterins of three different concentrations prepared from *S. paratyphi B* (previously called *S. schottmuelleri*), which is one of the components of Ovine and Swine mixed bacterins, and *S. choleraesuis* which is used in swine mixed bacterins were available in our laboratory. Mice were vaccinated with these bacterins by the established procedure and challenge inoculated with the *S. typhimurium* challenge culture. A bacterin prepared from

S. paratyphi B would protect as well as one prepared from *S. typhimurium* against a *S. typhimurium* challenge inoculation in mice.

S. choleraesuis bacterin produced some protection but much less than either *S. typhimurium* or *S. paratyphi B*.

Discussion and Conclusion

The investigations that were made established that calves could be experimentally infected with clinical Salmonellosis in a reproducible manner, and that calves vaccinated with a forminalized, adjuvanted bacterin of sufficient antigenic concentration will protect calves against clinical signs and death due to *S. typhimurium* infection.

It also indicated that mice can be used in a laboratory assay method for determining the potency of Salmonella containing bacterins using death as the criterion of infection. This assay method compares favorably with the potency of a bacterin in calves and provides a means for evaluating these bacterins on a serial to serial basis.

S. paratyphi B, *S. enteritidis*, and *S. choleraesuis* bacterins will protect mice in varying degrees against *S. typhimurium* infection. Cross protection between other antigenically related serotypes may be possible and is under investigation.

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STREPTOCOCCUS

RESPONSE OF VACCINATED SWINE TO GROUP E STREPTOCOCCUS EXPOSURE. Isaki, L. S., Bairey, M. H. and Van Patten, L. K. The Cornell Veterinarian, Vol. 63: No. 4, Oct. 1973, pp. 579-588. (Abstract of Published Report)

The response of swine to an avirulent group E *Streptococcus* vaccine given orally and intramuscularly was compared to the response of nonvaccinated swine. All pigs were exposed to virulent group E *Streptococcus* 21 days following vaccination. Animals were killed and necropsied 14 to 21 days following exposure.

All orally vaccinated pigs were protected as determined by the absence of abscesses after exposure, whereas 13 of 18 pigs given the vaccine intramuscularly, and 15 of 18 pigs not vaccinated had abscesses from which group E *Streptococcus* was isolated.

Serums, salivas, and lymph node extracts were assayed using precipitation, hemagglutination, and bacterial cell agglutination tests. Precipitating antibodies were not detected. Hemagglutinating antibodies were detected in all three fluids tested but were not correlated with the lack of abscessation following exposure. Antibodies that corresponded with protection were detected by bacterial cell agglutination in serums and lymph node extracts.

VESICULAR STOMATITIS

THE APPLICATION OF THE MICROTITER NEUTRALIZATION TEST FOR THE DETECTION OF ANTI-BODIES TO VESICULAR STOMATITIS VIRUSES. Wessman, S. J. (Project Report)

Summary

Serums were tested for antibodies to vesicular stomatitis by both egg and microtiter cell culture neutralization tests. Significant differences in serum antibody titers were not detected. The microtiter test was adopted as a replacement for the egg neutralization test because of its ease of use, speed, lower cost and reduced space and personnel requirements.

Introduction

Neutralization tests (NT) for the presence of antibodies against vesicular stomatitis virus (VSV) have been performed in eggs *(2, 3, 6,)*.[1] As demand for additional testing increased, particularly for certification of swine for export, it was evident that labor and supply costs would increase sharply, or a more efficient method of testing the samples would have to be developed. Microtitration procedures were already in use in the diagnostic virology section, VSL for detection of antibodies to bovine virus diarrhea, infectious bovine rhinotracheitis and parainfluenza-3 *(1)*. It was decided to attempt to develop a microtitration system for VSV, which would allow more flexibility in testing, while reducing laboratory and supply costs.

Materials and Methods

Production of Antigens.—Antigen production for use in the egg embryo NT has been described *(2, 3, 6)*. Antigen for the microtiter NT was produced by serially passaging the Concan strain of New Jersey type VSV and the San Juan strain of Indiana type VSV in the PK-15 cell line. The virus was grown in 250 ml. disposable plastic flasks[2] of 48-hour PK-15 cells. The maintenance medium was decanted, 2.0 ml. of a virus suspension was placed directly upon the cells, and the cultures were incubated at 37° C. for 30 minutes. The virus suspension was decanted and 30 ml. of Eagle's F-15 medium with antibiotics (200 units/ml. of penicillin, 0.2 mg./ml. of strptomycin, 0.2 mg./ml. of kanamycin and 0.005 mg./ml. of amphotericin B) containing 4 percent specific-pathogen-free (SPF) swine serum was added to each flask and the cultures were incubated at 37° C. Cultures were checked for evidence of cytopathic effect (CPE) at 24, 32, and 48 hours. When the CPE was observed to be 75 to 80 percent, the cultures were frozen at -90° F. Following two cycles of freeze-thrawing, the virus suspension was centrifuged at approximately 700Xg for 20 minutes. The supernate was bottled in 1 ml. aliquots and frozen at -90° F.

Test Procedures.—Virus titrations were performed in both Leighton tubes and disposable microtiter plates[2] (c $1/2 \times 5''$, rigid, sterile, with 96 flat-bottom wells) using PK-15 cells. Leighton tubes containing coverslips with

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[1] Numbers in parentheses refer to References at the end of this report.
[2] Falcon, Division of Becton, Dickinson & Company, Oxnard, CA.

confluent layers of 48-hour cells were inoculated with 0.1 ml. does of tenfold serial dilutions prepared from each virus. Four tubes were inoculated per dilution, and incubated at 37° C. Parallel titrations were carried out in microtiter plates using 0.025 ml. of Eagle's F-15 with antibiotics, 0.025 ml. of the serial tenfold dilutions of virus, and 0.05 ml. of a suspension of PK-15 cells per well, sufficient cells were added to form a monolayer in 24 to 48 hours. Each well was then overlaid with two drops of mineral oil and incubated at 37° C. in a 5 percent CO_2 atmosphere.

Serums from convalescent and hyperimmunized animals, field cases and normal animals were tested. Neutralization tests were performed in eggs as previously described (6), except that a fourfold dilution scheme was used. Serums were inactivated at 60° C. for 20 minutes. For the microtiter NT serial twofold dilutions of serum (0.025 ml. amounts) were carried out by an automatic diluter,[3] using Eagle's F-15 with antibiotics as diluent. Twenty-five thousandths ml. of virus, previously titered to contain 100 to 200 $TCID_{50}$, was added to each dilution and the serum-virus mixtures were allowed to neutralize at room temperature for 1 hour. Five hundredths ml. volumes of PK-15 cell suspensions, approximately 5,000 cells, were placed in each well, overlaid with mineral oil and the completed test incubated at 37° C. in a 5 percent CO_2 atmosphere. Known positive and negative serums, as well as virus titrations, were included as controls.

Turbid serums or serums found toxic to cell cultures were absorbed by mixing with a 25 percent acid-washed kaolin[4] suspension. After 15 minutes adsorption, the mixture was clarified at 1,500 RPM for 15 minutes, in a PR_2 centrifuge,[5] using a #253 head, and then used in the test.

Results

Egg embryo deaths were recorded as previously described (2, 6). The microtiter NT was read at 48 hours using an inverted microscope.[6] The tube NT was also read at 48 hours. Endpoints were considered to be the last dilution where virus was neutralized as evidenced by the absence of a cytopathic effect. Titers for all tests were calculated by the method of Kärber (4). Virus titers are shown for all three tests, with a conversion to a standard volume (table 1). When converted to a 1 ml. standard, titers varied from 1.3×10^6 to 5.5×10^6 $TCID_{50}$ for New Jersey type VSV, and from 1.3×10^7 to 1.8×10^7 $TCID_{50}$ for Indiana type VSV.

Table 1.—*Virus titrations for all methods tested, with titers expressed both by test dose and conversion to standard volume*

Virus	Test	Titer (test dose)	Titer (per ml.)
NJ	Microtiter	6.4×10^4 $TCID_{50}$/0.025 ml.	2.6×10^6 $TCID_{50}$
NJ	Macro (tube)	5.5×10^5 $TCID_{50}$/0.1 ml.	5.5×10^6 $TCID_{50}$
NJ	Egg	2.0×10^5 ELD_{50}/0.15 ml.	1.3×10^6 ELD_{50}
IND	Microtiter	4.0×10^5 $TCID_{50}$/0.025 ml.	1.6×10^7 $TCID_{50}$
IND	Macro (tube)	1.8×10^6 $TCID_{50}$/0.1 ml.	1.8×10^7 $TCID_{50}$
IND	Egg	2.0×10^6 ELD_{50}/0.15 ml.	1.3×10^7 ELD_{50}

[3] Cooke Engineering, Alexandria, VA.
[4] Fisher K5, Fisher Scientific Co., Fairlawn, NJ.
[5] International Equipment Co., Boston, MA.
[6] American Optical Co., Buffalo, NY.

The majority of samples received during this period were from animals requiring a neutralization test for VS for export certification. Two hundred and eighty-three samples for export, tested by both microtiter and egg serum neutralization, were negative on both tests. For comparison of the two tests on positive serums it was necessary to use samples from animals which had been hyperimmunized and from which serum had been collected (See tables 2, 3, and 4).

Replicate testing of these serums showed that there was good agreement between the two tests, as previously reported (8). Sixty percent (12/20) showed essentially identical titers, while titers of the remaining samples varied by less than a two or fourfold dilution, depending upon the titration scheme. The average titer for all samples tested by the egg neutralization test was 2.03 while the titer on the same serums using the microtiter test was 2.10, with the titer expressed as the logarithm of the reciprocal of the highest serum dilution neutralizing 100 ELD_{50} or 100 $TCID_{50}$ of virus. The microtiter NT was based on a twofold dilution series (1:8, 1:16, . . . 1:512), while the egg NT had been modified to utilize a fourfold dilution scheme (1:8, 1:32, 1:128, 1:512). Occasionally a 1:16 dilution was used in the egg NT, resulting in a dilution series of 1:16, 1:64, 1:256, and 1:1024. Both tests were read using 50 percent endpoint. Differences in the dilution schemes probably contributed to titer variations.

Table 2.—Egg neutralization test results

| Serum | Test # | Titer[1] | | | Average titer |
| | | Test numbers | | | |
		1	2	3	
C-3908		>2.1	2.4	2.4	2.50
73-7787-1		>2.1	1.8	1.8	1.80
73-7787-5		>2.1	2.4	2.7	2.55
73-7994-72		>2.1	2.4	2.7	2.55
73-7994-73		>2.1	2.4	2.1	2.25
73-7995-76		>2.1	1.8	1.5	1.65
73-8000-47		>2.1	2.4	2.1	2.25
73-8004-6		>2.1	2.4	2.4	2.40
73-8005-26		>2.1	2.4	2.1	2.25
73-8159-267		>2.1	2.4	2.4	2.40
73-8166-260		1.5	1.2	1.5	1.40
73-8166-261		>2.1	1.8	1.5	1.65
73-8166-262		>2.1	2.4	2.4	2.40
73-8167-226		>2.1	2.4	2.1	2.25
73-8167-230		>2.1	2.4	2.1	2.25
73-8167-235		0.9	1.2	0.9	1.00
73-8167-241		0.9	1.2	0.9	1.00
73-8169-280		>2.1	2.4	2.1	2.25
73-8169-281		>2.1	2.4	2.1	2.25
73-8170-2		>2.1	1.8	1.5	1.65
Normal		N 0.9	N 0.9	N 0.9	N 0.9
Average titer		2	2.10	1.96	2.03

[1] Titers expressed as logarithm of reciprocal of last serum dilution neutralizing 100 ELD_{50} of virus.   N.T. = Not Tested.
[2] As a majority of serums were not tested to endpoint, no average titer was computed.
\> - Greater than.

Table 3.—Microtiter neutralization test results

Serum	Test #	Titer[1]				Average titer
		Test numbers				
		1	2	3	4	
C-3908		2.7	2.7	>2.7	>2.7	2.70
73-7787-1		2.1	2.1	2.1	N.T.	2.10
73-7787-5		2.7	2.4	2.4	>2.7	2.70
73-7994-72		2.4	2.4	2.4	>2.7	2.48
73-7994-73		2.4	2.7	2.7	2.4	2.55
73-7995-75		1.2	1.5	1.5	1.2	1.38
73-8000-47		2.4	2.4	2.4	2.4	2.40
73-8004-6		>2.7	>2.7	>2.7	>2.7	>2.70
73-8005-26		2.1	2.1	2.1	2.1	2.18
73-8159-267		2.4	2.4	2.4	2.4	2.40
73-8166-260		1.2	1.2	1.2	1.2	1.28
73-8166-261		1.5	1.8	1.8	1.8	1.73
73-8166-262		2.4	2.4	2.4	2.4	2.40
73-8167-226		1.8	1.8	1.8	1.8	1.80
73-8167-230		2.4	2.4	2.4	2.4	2.40
73-8167-235		0.9	0.9	0.9	0.9	0.90
ʹ75-8167-241		1.2	1.2	1.2	1.2	1.20
73-8169-280		2.4	2.4	2.7	2.7	2.55
73-8169-281		2.4	2.1	2.1	2.1	2.25
73-8170-2		1.8	1.8	1.8	1.8	1.80
Normal		N 0.9	N 0.9	N 0.9	N 0.9	N 0.9
Average titer		2.05	2.14	2.06	2.09	2.10

[1] Titers expressed as logarithm of reciprocal of last serum dilution neutralizing 100 TCID of virus. N.T. = Not Testes.

> = Greater than.

Table 4.—Comparison of average titers

Serum	Egg NT Titer[1]	Microtiter NT Titer[2]
C-3908	2.50	2.70
73-7787-1	1.80	2.10
73-7787-5	2.55	2.70
73-7994-72	2.55	2.48
73-7994-73	2.25	2.55
73-7995-76	1.65	1.38
73-8000-47	2.25	2.40
73-8004-6	2.40	>2.70
73-8005-26	2.25	2.18
73-8159-267	2.40	2.40
73-8166-260	1.40	1.28
73-8166-261	1.65	1.73
73-8166-262	2.40	2.40
73-8167-226	2.25	1.80
73-8167-230	2.25	2.40
73-8167-235	1.00	0.90
73-8167-241	1.00	1.20
73-8168-280	2.25	2.55
73-8169-281	2.25	2.25
73-8170-2	1.65	1.80
Normal	N 0.90	N 0.9
Average titer	2.03	2.10

[1] Titers expressed as logarithm of reciprocal of last serum dilution neutralizing 100 ELD_{50}.

[2] Titers expressed as logarithm of reciprocal of last serum dilution neutralizing 100 $TCID_{50}$ of virus.

> = Greater than.

Discussion

Factors influencing virus infectivity have been studied by other workers *(5)* and Rosenthal and Schechmeister have compared microtiter sensitivity to that of the plaque test *(7)*. The twofold dilution scheme appears more sensitive than the fourfold series because the interval between dilutions is smaller. For this reason and the others cited below it was decided to use the microtiter VS SN test for all diagnostic work. Using tissue culture rather than eggs permits testing 4 days per week rather than once, eliminating dependence upon a source of 8-day old embryonated eggs. It is also less expensive to discard a few flasks of tissue culture than to discard many dozen eggs if samples do not arrive. A cost ratio of approximately 1:70 for the microtiter NT versus the egg NT can be computed, based on $1.75 for one Falcon flask of cell culture for testing nine samples plus controls at seven dilutions, versus $111.00 for 30 dozen eggs for testing the same number of samples at only three dilutions. Laboratory space can also be saved by eliminating the need for an egg incubator. The test can be prepared and read in less time in cell culture than in eggs for the same number of samples. A possible disadvantage is the greater instance of toxicity using the microtiter system, however, the kaolin treatment appears to correct this problem. Basically, more samples can be tested with economy using the microtiter system.

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EXPERIMENTAL INFECTION OF COTTON RATS (SIGMODON HISPIDUS) WITH INDIANA SUBTYPE I VESICULAR STOMATITIS VIRUS. Jenney, E. W., Brown, C. and Matthews, P. J. (Project Report)

Summary

Cotton rats, *Sigmodon hispidus*, were inoculated with vesicular stomatitis virus (VSV) (suspension of bovine tongue vesicle) by two routes and virus isolations were attempted to determine the possible involvement of cotton rats in the epizootiology of Indiana subtype I virus.

One nonspecific death occurred with the Indiana type when 35 cotton rats were inoculated intranasally, and none died out of 24 inoculated subcutaneously. All surviving rats produced antibodies. Virus was isolated from brain tissue of 8 of 10 rats inoculated intranasally and euthanatized at 2 to 6 days post-inoculation (DPI). Virus was isolated from the lungs of six of six rats euthanatized 2 to 4 DPI. Following subcutaneous inoculation, virus was isolated from the spleen and lung of one of two killed at 1 DPI, the spleen of one of five killed at 2 DPI and the spleens and kidneys of two or four killed at 3 DPI. None of 15 rats given Indiana type virus orally produced antibodies to Indiana virus. Neurotropic properties of Indiana type virus were suggested as a result of the isolations.

Introduction

The mite genus, *Gigantolaelaps*, from which workers at the Trinidad and Belem Rockefeller Foundation Virus Laboratories isolated Cocal VSV (Indiana subtype 2) *(6, 7, 8),*[1] was found on terrestrial rice rats, *Oryzomys*, and on cotton rats *(10)* in the southern United States. These mites had been collected from *Oryzomys*, *Heteromys* (spiny pocket mouse) and *Zygodontomys* (cane mouse), all of which were found to be involved in the 1961 outbreak of the Cocal strain of VSV at Trinidad, W.I. Livestock were not present in this area. Skinner has demonstrated the susceptibility of cotton rats to intracerebral inoculation with VSV *(11)*. In a preceding paper we reported on experimental New Jersey VSV infections in cotton rats *(5)*.

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[1] Numbers in parentheses refer to References at the end of this report.

In an effort to determine its potential role in the epizootiology of VSV laboratory propagated cotton rats were inoculated intranasally (IN), subcutaneously (SC) or given feed covered with a suspension of Indiana (Ind) type vesicular stomatitis virus (VSV).

Material and Methods

Cotton Rats.—The source, housing and handling of the cotton rats were described in a previous paper (5).

Virus Strain.—The Ind type virus, subtype 1, Cannon City strain, was the first passage from udder vesicles obtained from an infected cow in Colorado during 1964. The inoculum used was bovine tongue epithelium obtained 3 days post-inoculation (DPI). Preparation of the 5 percent suspension used for inoculation of the rats has been described (5).

Infectivity titer of the 5 percent virus suspension was determined using five 8-day embryonated chicken eggs per virus dilution. Fifty percent mortality was obtained in eggs with 0.1 ml of the 10^{-4} dilution of virus suspension. This dosage was used for the intranasal (IN) and subcutaneous (SC) routes of inoculation.

Inoculation.—(Intranasal Route) Thirty-five cotton rats were inoculated IN (table 1) and held 6 to 33 days for observation. Virus suspension was slowly introduced into the nares of each anesthetized cotton rat with a syringe and needle. Twelve inoculated animals were euthanatized (2 per day from DPI 2-7) for virus isolation (table 2).

Table 1.—*Survival and antibody development in cotton rats exposed to Indiana vesicular stomatitis virus by various routes*

Method of inoc.	Age	No. deaths/ no. inoc.	Survival range (days)	Total deaths by route/ total	Survivors with titers/ total survivors
IN[1]	2 mo.	1/30	14 (one death)[2]		29/29
	Adult	0/5	No deaths	1/35[2]	5/5
SC[1]	2 mo.	0/21	No deaths		21/21
	Adult	0/3	No deaths	0/24	3/3
Oral	2 mo.	0/10	No deaths	0/10	0/10

[1] IN - Intranasal, SC - Subcutaneous.
[2] Negative on virus isolation attempts from tissues; excluded from virus deaths.

Subcutaneous Route.—Twenty-four cotton rats were inoculated SC and held for observation. A group of 19 cotton rats was inoculated subcutaneously and sequentially killed for virus isolation. Four were killed per day 1 to 3 DPI and two per day 4 to 7 DPI.

Oral.—Ten cotton rats were fed Ind VSV on their food. A "dose" of 0.2 ml of the virus suspension per cotton rat was poured over ground feed in petri dish halves and placed in their cages for 24 hours. Rats in each cage ate between one-half and three-fourths of their food containing the virus.

Contact Controls.—One normal control was placed with each two animals at the time of inoculation, except for one group of 10 IN and 8 SC in which controls were added one DPI.

Virus Isolation.—Oral swabs were taken 2 to 5 DPI from rats inoculated IN; no swabs were taken from those inoculated SC. Disposition of dead rats and preparation of tissues for virus isolation has been described (5). Six 8-day embryonated chicken eggs were inoculated with each sample. Allantoic and amnionic pooled fluids from each dead egg were tested by the complement fixation test (3) to confirm the presence of the virus.

Serology.—As previously described (5) surviving animals were usually bled for serum 2 to 4 weeks following exposure. Neutralization (Nt) tests were performed at 1:20 and 1:80 on the serum of each rat that was killed. Eighteen serums were titrated by the CF test (4).

Table 2.—Virus isolations from cotton rats inoculated with Indiana vesicular stomatitis

Route of inoc.	Status and number days PI[1]	Plasma	Brain	Lung	Kidney	Bladder	Liver	Spleen	Feces	Oral swabs
IN	K 2 - 4	0/6[2]	6/6	6/6	0/6	0/6	--	1/6	0/6	--
	K 5 - 7	0/6	2/6	0/6	1/6	0/6	--	0/6	0/4	--
	A 2 - 3	--	--	--	--	--	--	--	--	5/9
	A 4 - 5	--	--	--	--	--	--	--	--	0/8
SC	K 1 - 7	0/18[3]	0/19	1/18	1/18	0/19	0/7	4/18	0/12	--

[1] A-Alive; K-Killed.
[2] In all cases the number of positive virus isolations is listed over the number of virus isolation attempted.
[3] Seven buffy coat samples were also negative.

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Results

Intranasal Trial.—Virus was isolated from five of nine oral swabs taken 2 and 3 DPI, but not on the fourth and fifth DPI (table 2). Only one death occurred among 35 cotton rats inoculated IN and held for observation. The death occurred 14 DPI and virus was not isolated from the rat's tissues. All survivors developed antibodies. In the group of 12 animals sacrificed for virus isolation, virus was isolated from the brain tissue of the 6 rats killed 2 to 4 DPI, 1 of 2 euthanatized on each of 5 and 6 DPI and none of the 2 killed 7 DPI. In addition, virus was isolated from lung specimens obtained from the rats sacrificed 2 to 4 DPI, but virus was not isolated from lung tissue of the rats sacrificed on 5, 6, and 7 DPI. Virus was isolated from the spleen of one rat sacrificed 2 DPI and from kidney tissue of one rat euthanatized 6 DPI.

Subcutaneous Trial.—Virus was isolated from the spleens of 4 of the 11 cotton rats euthanatized on the first, second, and third DPI. Virus was isolated from the lung of one rat at 1 DPI and from the kidney of another at 3 DPI. Other tissues from the 19 rats were negative for virus isolation (table 2). All of the 24 rats survived and developed antibodies (table 1).

Oral Trial.—None of 10 animals given Ind VSV on their feed developed signs of infection nor antibodies against the disease.

Contact Controls.—There were no deaths in the contact control rats but 11 of 18 caged with IN inoculated animals developed antibodies (table 3). The proportion of controls that became infected when added 1 DPI was the same as those when the controls were present at time of inoculation. Antibodies were not detected in the eight controls placed with the SC group.

Table 3.—Transmission of Indiana vesicular stomatitis virus to contact controls—following inoculation of cotton rats by two routes

Exposure group	No. deaths/no. exposed	No. with antibodies/ no. exposed
Intranasal	0/18	11/18
Subcutaneous	0/8	0/8

Serology.—Indiana type Nt titers of 1:80 were detected in most surviving animals.

Seven of 8 IN inoculated rats had CF titers of 1:80, and of 1:40. Seven Nt titers of this group were 1:80 and 1 was 1:40. The 1:40 titers for CF and Nt were in rats bled at 7 DPI. Two CF titers of 1:80, at 7 DPI, were accompanied by heterologous titers of 1:10 against New Jersey type VSV. Two rats had heterologous titers of 1:5 and the serums of four were type specific.

Discussion

Only one death occurred in the 35 cotton rats inoculated IN. Virus was not isolated from tissue of this rat so death did not appear to have been caused by the virus. In rats inoculated and killed sequentially, Ind VSV was isolated from brain tissues at 2 through 6 DPI. The low mortality was in sharp contrast to 24 to 25 deaths with New Jersey IN and 12 to 16 controls that died following contact to IN inoculated rate (5); therefore, a low mortality would be expected in a native cotton rat population infected with Indiana I type in contrast to an expected die-off with NJ type virus.

One of the striking findings was the high frequency with which virus was isolated from brain tissue of cotton rats following IN inoculation. Although only one death occurred, virus was isolated from 8 to 10 rats killed 2 to 6 DPI. Miyoshi Harter and Hsu (9) described neuropathology produced by Indiana type VSV in young mice inoculated

intracerebrally and intranasally. Their fluorescent antibody and virus isolation studies indicated direct invasion of the olfactory bulb and structures following intranasal instillation. Vesicular stomatitis virus appeared to multiply in the cytoplasm of neurons.

Following the SC inoculation of 18 rats, the virus was isolated from the spleens of four animals, the lung of one animal and the kidneys of one animal. Virus was not isolated from the brain tissue of these 18 rats. This was an indication that the reticuloendothelial system was active in restraining the virus *(1)*. Most of the rats developed CF and Nt titers of at least 1:80 following exposure to the virus. All surviving animals inoculated IN or SC developed VS neutralizing antibodies. Results indicate that cotton rats infected under natural conditions could be detected by serology; subclinical infection could occur.

Survival of the cotton rats inoculated with Indiana VSV parallels Jonkers' results with the closely related *Oryzomys*, rice rat, following inoculation with Ind subtype 2, Cocal virus *(7)*.

Eleven of 18 uninoculated controls in contact with intranasally exposed cage mates developed antibodies (table 3). This contrasts with the total lack of antibody developed following the feeding of VSV and contact with SC exposed animals. With the high rate of recovery of VSV from the lungs following exposure to both NJ and Ind type by IN inoculation, the possibility of aerosol transmission among animals in close confinement should be considered.

In 1945 Frank, Appleby, and Seibold *(2)* reported deaths following intracerebral (IC) inoculation of horses, cattle and sheep with a mouse brain passaged strain of New Jersey VSV. However, survival occurred following IC inoculation with mouse brain passaged Indiana type VSV. In our work only bovine tongue passaged virus isolated from field cases was used. Ind I VSV was found to be less virulent than New Jersey VSV following In and SC inoculations of cotton rats.

It appeared from the preceding results that if cotton rats are naturally infected with Indiana I VSV by the respiratory route they could produce antibodies, it would be a mild infection and they could transmit it via the respiratory route. It appears from the IN controls that they spread Indiana VSV by respiratory droplet infection. There was no spread from subcutaneously inoculated cotton rats and as no antibodies were found following oral exposure, these routes do not appear to be routes of spread in nature. The intravenous route simulating the bite by blood sucking insects has not been tried. Viremia has rarely been demonstrated with VS yet Indiana VSV was isolated from nasal swabs on DPI 2 and 3 following IN exposure. Contact controls placed with the IN exposed cotton rats developed antibodies yet those given virus orally remained negative. The respiratory route was the only potential route tested and its feasibility for transmission in nature is questionable as relatively close confinement and a high population density would seem necessary.

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EXPERIMENTAL INFECTION OF COTTON RATS (SIGMODON HISPIDUS) WITH NEW JERSEY TYPE VESICULAR STOMATITIS VIRUS. Jenney, E. W., Brown, C., and Matthews, P. J. (Project Report)

Summary

Cotton rats, *Sigmodon hispidus*, were inoculated with vesicular stomatitis virus (suspension of bovine tongue vesicles) by two routes and virus isolation was attempted to determine the possible involvement of cotton rats in the epizootiology of New Jersey (NJ) type virus. Cotton rats inoculated intranasally died (24 to 25) as did most of those inoculated subcutaneously (12 to 16). Virus was isolated from the brains (26 to 27), lungs (2 to 27) and kidneys (4 to 27) of fatal cases. Cotton rats were very susceptible to infection by the respiratory route and the five survivors produced antibodies. Ten rats given virus suspension orally remained healthy and without serological response.

Introduction

The distribution of cotton rats roughly covers the vesicular stomatitis (VS) enzootic areas of the United States. It is one of the most abundant mammals in areas of the Americas where VS occurs. Rodents have been found to be naturally infected with the Cocal subtype of VS virus in some countries. Unusually heavy populations of cotton rats were present in southern Texas during the 1949 and 1959 outbreaks of NJ type VS (Lewis Seay and Richard Zirkle personal communication, 1968, USDA, APHIS, Room 301, 702 Colorado Street, Austin, TX 78701). The mite genus *Gigantolaelaps*, from which Cocal VSV (Indiana subtype 2) *(7, 8, 9),*[1] has been isolated, is found on terrestrial rice rats, *Oryzomys*, and on cotton rats *(13)* in the southern United States. *Hetermoys* (spiny pocket mouse) and *Zyodontomys* (cane mouse) were found to be involved along with *Oryzomys* in the 1961 Trinidad outbreak. The Cocal virus enzootic occurred in an area void of domestic animals *(7, 9).* Skinner demonstrated the susceptibility of cotton rats to intracerebral inoculation *(15).* Laboratory propagated cotton rats were inoculated intranasally (IN), subcutaneously (SC) or given food covered with a virus suspension of New Jersey (NJ) type vesicular stomatitis virus (VSV) in an effort to determine their potential role in the epizootiology of VSV.

Material and Methods

Cotton Rats—Young breeding stock was obtained from the colony at the National Center for Disease Control in Atlanta, GA. One-to three-month-old progeny and adult females were used.

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[1] Numbers in parentheses refer to References at the end of this report.

Table 1.—*Survival and antibody development in cotton rats exposed to New Jersey vesicular stomatitis virus by various routes*

Method of inoc.	Age	No. deaths/ no. inoc.	Survival range (days)	Total deaths by route/ total inoc.	Survivors with titers/ total survivors
IN[1]	2 mo.	19/19	4-7		
	Adult	5/6	4-8	24/25	1/1
SC	2 mo.	9/10	6-13		
	Adult	3/6	5-6	12/16	4/4
Oral	2 mo.	0/10	No.deaths	0/10	0/10

[1] IN - Intranasal, SC - Subcutaneous.

Subcutaneous—Sixteen cotton rats, 10 young and 6 adult, were inoculated SC with New Jersey VSV using 0.1 ml. of the virus suspension.

Oral—Ten cotton rats were routinely fed "Laboratory Chow"[2] pellets. For the oral exposure "Laboratory Chow" pellets were ground to make it more absorbent for the VSV inoculum. A "dose" of 0.2 ml. of the virus suspension per cotton rat, 0.4 ml. per cage, was poured over ground feed in a petri dish half and placed in their cages for 24 hours.

Contact Controls—One normal contact control was placed with two inoculated animals at the time of their inoculation, except for one group in which controls were added 1 day post-inoculation (DPI). Controls were added to three cages with orally exposed animals at 2 DPI.

Animals were fed and examined daily. Dead or killed animals were placed in individual plastic bags, identified, frozen, and stored at −68° C. until virus isolation was attempted.

Exposed rats were housed in plastic mouse cages with metal tops designed to hold water bottles and pelleted food. These cages were placed in banks of plastic small animal isolation cages with negative air control. Two inoculated rats and a control were placed in each mouse cage.

Cotton rats were handled by placing a 6-inch length of 3-inch diameter black plastic pipe into the cage. The rats readily entered the pipe and were dumped into a 3-pound coffee can containing ether on cotton. All rats were anesthetized prior to inoculation or bleeding. Due to the ability of cotton rats to jump 2 feet vertically, mouse cages were opened at the bottom of large galvanized cans or in cardboard cartons with sides at least 2½ feet high.

Virus Strain—The NJ type, Atlanta strain, was isolated in 1962 from an outbreak at Atlanta, GA. The source of all NJ type virus used was tongue epithelium from a bull harvested 40 hours following intradermallingual inoculation. This was the third bovine passage from the original specimen.

Vesicular epithelium was ground by mortar and pestle and diluted to a 5 percent suspension with TRIS[3]-buffered-tryptose broth containing 10,000 units of streptomycin sulfate per ml. The suspension was centrifuged and the supernatant stored at −68° C.

Infectivity titer of the virus suspension was determined in 8-day embryonated chicken eggs. The 50 percent mortality endpoint of the inoculum was obtained with 0.1 ml. of the 10^{-5} dilution.

Inoculation(—Intranasal)—Twenty-five cotton rats, 19 young and 6 adults, were inoculated IN with NJ type VSV (table 1). One-tenth ml. of virus suspension was slowly introduced into the nares of each anesthetized cotton rat with a syringe and needle and the rat was held to observe the course of the disease. In a second experiment eight cotton rats inoculated IN were killed sequentially (two per day from DPI 2-5) and tissues were collected for virus isolation.

Virus Isolation—Oral swabs for virus isolation were taken from ten rats following inoculation. Swabs were placed in fresh TRIS-buffered-tryptose broth on 2 to 6 DPI and frozen. The swabs were depressed against the tube and removed leaving the broth of virus isolation. Six eggs were inoculated in the chorioallantoic sac using 0.15 ml.

[2] Laboratory Chow from Teklad Mills, Winfield, IA.
[3] Trizma Base No. T-1503 from Sigma Chemical Co.

doses. Dead animals were thawed and selected tissues removed using separate sterile foreps and scissors. All tissues were ground with a mortar and pestle in a biological hood. Tissues were diluted in 2 to 3 ml. TRIS-buffered-tryptose broth blanks containing 5 mg. of streptomycin sulfate and 5,000 units of potassium penicillin G per ml. Tissue suspensions were centrifuged and the supernates frozen and stored at $-68°$ C until they were inoculated. Six 8-day embryonated chicken eggs were inoculated per sample by the allantoic route with 0.1 ml. doses of tissue suspension. The chorioallantoic and amnionic membranes were ruptured and the allantoic and amnionic fluids of each dead embryo were aspirated as a pool and tested by the complement fixation (CF) test (6) to confirm the presence and type of virus isolation from each tissue.

Serology—Surviving animals were bled 2 to 4 weeks following exposure and the serum frozen. Some adults were bled after only 1 week. Neutralization tests (Nt) were performed on each serum sample. Netutralization tests were performed with VS virus prepared from egg membranes diluted in their allantoic-amnionic fluid as previously described (5). One-half ml. of a virus dilution containing 100 ELD_{50} (100 doses of a virus dilution that killed 50 percent of the embryos per 0.15 ml. dose) wqs added to 0.5 ml. of the 1:10 and 1:40 serum dilutions in sterile, stoppered, 13×100 mm. tubes and mixed. After 30 minutes incubation at $22°$ C; five 8-day embryonated chicken eggs were inoculated by the allantoic route with 0.15 ml. doses of the serum-virus mixture. One egg per serum was inoculated with equal volumes of the 1:10 dilution of serum and diluent as a serum control. TRIS-buffered-tryptose broth, pH 7.6, was used as diluent for both serum and virus. Five micrograms of streptomycin sulfate and 5,000 units of penicillin G per ml. was used in the virus diluent for the test and in the serum controls. The embryonated eggs were incubated at $35°$ C. The 50 percent mortality endpoints (Nt titers) were determined by the Karber (10) method from embryos that died 16 to 72 hours following inoculation. Serums were not titrated for endpoints but only to determine if the individual animals had become infected. Heterologous type Nt were not performed.

Serums from three cotton rats were titrated by the CF test (5) to determine the presence of complement-fixing antibodies in convalescent cotton rat serum.

Results

Intranasal Trial—Twenty-four of 25 cotton rats inoculated IN died between 4 and 8 DPI. The remaining animal survived and developed neutralizing antibodies (table 1).

Results of virus isolation from this group and the eight rats killed sequentially following infection are also given (table 2). Although a daily bleeding and a sacrifice of two cotton rats per day was scheduled for the IN group, all were dead on the fifth DPI except two which were sick and sacrificed. Virus was isolated from 22 of 23 brains of rats which died or were killed following IN inoculation. Virus was also isolated from the lungs of 7 to 22, kidneys of 6 to 23, spleens of 1 to 23, and from the blood plasma of 1 bled 2 DPI. Tissues from the 24th dead rat were apparently not examined for the presence of virus in the tissues.

Ten percent suspension was prepared from the brains of four cotton rats found dead of New Jersey VSV 4 days after IN inoculation. Infectivity titers of their brain suspensions in 8-day embryonated chicken eggs were 10 (4) and 10 (5) per 0.1 ml. Affected animals became inactive before death.

Subcutaneous Trial—Twelve of 16 cotton rats inoculated SC died between 5 and 13 DPI (table 1) and the 4 survivors developed antibody titers. Virus was isolated from the brain of 12 rats and from the kidneys of one (table 2).

Oral Trial—From one-half to all of the food in the various cages was eaten within 24 hours. No sickness was observed and all 10 serums were negative 30 DPI.

Oral Swabs—New Jersey virus was isolated from one of six swabs taken 2 days following IN inoculation (table 2). Virus was not isolated from 12 swabs taken 3 to 4 DPI.

Contact Controls—A summary of the mortality and antibody production of the contact controls was made (table 3). Twelve of the 16 controls died and 1 of the 4 survivors developed antibodies in the IN exposed group. The five controls added 1 DPI were among those that died, other controls were present from time of inoculation. Three of the survivors were from the adult groups. None of the 11 controls died and only 1 developed an antibody titer in the SC exposed group.

Table 2.—Virus isolations from cotton rats inoculated with New Jersey vesicular stomatitis

Route of inoc.[1]	Age	Status and number days PI[2]	Plasma	Brain	Lung	Kidney	*Bladder	Spleen	Feces	Testicle	Oral swabs
IN	2 mo	D 5 - 6	--	10/10[3]	2/10	2/10	--	0/10	--	--	--
	2 mo.	K 2 - 5	1/8	8/8	5/7	3/8	0/8	1/8	0/8	0/2	--
	2 mo.	A 2	--	--	--	--	--	--	--	--	1/6
	2 mo.	A 3 - 4	--	--	--	--	--	--	--	--	0/12
	Adult	D 4 - 6	--	4/5	0/5	1/5[4]	--	0/5	--	--	--
SC	2 mo.	D 6 - 13	--	9/9	0/9	1/9	0/8	0/9	--	--	--
	Adult	D 5 - 6	--	3/3	0/3	0/3[4]	--	0/3	--	--	--

[1] IN - Intranasal, SC - Subcutaneous.
[2] A-Alive; D-Dead; K-illled.
[3] Numerator/denominator—In all cases the number of positive virus isolation is listed over the number of virus isolations attempted.
[4] Bladder and kidney pooled.

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Table 3.—*Transmission of New Jersey vesicular stomatitis virus to contact controls following inoculation of cotton rats by two routes*

Method of inoc.	No. deaths/ no. exposed	Survival range days	No. survivors with antibodies/ no. survivors
Intranasal	12/16	5 - 9	1/4
Subcutaneous	0/11	No deaths	1/11

Serology—The rat that survived IN inoculation had a 1:20 Nt and a negative CF titer when bled 7 DPI. Of four which survived SC inoculation one had a 1:20 Nt and 1:80 CF titer at 7 days; three had 1:80 Nt titer when bled out at 7, 16 and 30 DPI. All 10 rats exposed orally and the 3 contact controls were negative.

Discussion

A higher mortality followed infection by the IN route (24 to 25) than with the SC route (12 to 16) of inoculation.

Two of four cotton rats tested had neutralizing and CF titers at 7 DPI.

One of the striking findings was the high frequency with which virus could be isolated from brains of cotton rats following their inoculation with VS virus. Virus was isolated from the brain of 34 to 35 rats following death.

Twelve of 16 control cotton rats became infected and died when exposed to cagemates inoculated IN with New Jersey VSV (table 3). This contrasts the total lack of infection following oral exposure to NJ virus and the low infection (1 to 11) in control rats placed in contact with SC inoculated rats. With the high rate of recoveries of VSV from lungs following infection with the NJ type by IN inoculation, it seems there was a possibility of aerosol transmission among animals in close confinement. This is supported by the high percentage of contact controls that died or developed antibodies when placed with IN infected animals. Antibody titers were not detected in any of the animals after oral exposure, and in only 1 of 11 controls placed with the animals inoculated SC.

New Jersey VS occurred in Texas during 1949 *(3)* and 1959 *(12)* concurrently with large populations of cotton rats *(1)*. During these outbreaks there was no evidence that the cotton rat population was infected. If the cotton rats had become infected with NJ type virus, a die-off should have occurred such as has been reported by Meyer. *(12)*

Encephalitis has been reported with New Jersey VS in three naturally occurring equine cases. *(14)* In 1945 Frank, Appleby, and Seibold *(2)* reported deaths following intracerebral (IC) inoculation of a mouse brain passaged strain of New Jersey VSV into horses, cattle and sheep. Their original New Jersey VSV strain, collected during 1926, had been passaged intracerebrally in mice and guinea pigs to encourage any neurotropic tendencies. In these experiments only bovine tongue passaged virus isolated from natural field cases was employed. New Jersey VSV was found to be virulent to cotton rats by IN or SC inoculation. Neutrotropic tendency of New Jersey type VSV was demonstrated. Cotton rat populations should be studied during VS outbreaks as they are potential reservoir hosts of New Jersey type VSV. Results of similar inoculations with Indiana I type VSV are being reported in a separate paper. *(4)*

Acknowledgment

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MISCELLANEOUS

BACTERIA ISOLATED FROM IMPORTED SALTED DUCK EGGS. Harrington, R., Jr., Hulse, D.C. and Ellis, E. M. Avian Diseases, Vol. 18, No. 2, April-June, 1974, pp. 240-242. (Abstract of Published Report)

Salted duck eggs imported from Taiwan were examined for bacteria. The following organisms were isolated: *Pseudomonas cepacia* (a pathogen of emerging importance to public health officials which is now under intensive study), *Alcaligenes metalcaligenes*, *A. recti*, *Micrococcus luteus*, *Pasteurella hemolytica*, and *Ps. aeruginosa*.